Clock Gene Dysregulation and Nuclear Receptor Expression in DSS-induced Colitis

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Table of contents

Chapter 1.

SUMMARY

CHAPTER 1. SUMMARY

1.1. Summary (German).

Zusammenfassung

Hintergrund und Zielsetzung: Bei entzündlichen Erkrankungen führt die Aktivierung des Immunsystems zu einer Zytokin-vermittelten Unterdrückung der Expression von Genen des zirkadianen Rhythmus (CisCG), z.B. die Gene Clock und Period und Transkriptionsfaktoren der proline and acidic-rich bZIP Familie D-site albumin promoter-binding protein (dbp), tyrotroph embryonic factor (tef) und hepatic leukemia factor (hlf) in myeloiden Zellen.

Kachexie is ein Energieverlustssyndrom angetrieben durch ein Ungleichgewicht zwischen Immunsystem und Metabolismus, welches sich vorallem in der letzten Phase der meisten chronisch entzündlichen Krankheiten wie z.B bei Krebsleiden manifestiert. Die Störung zeichnet sich durch extremen Gewichts-, Körperfett- und Muskelschwund aus, was zu Erschöpfung und einem erhöhten Risiko von Sekundärinfektionen führt. Letzeres wurde kürzlich der Anwesenheit von unreifen myeloiden Suppressorzellen (MDSCs) zugeschrieben.

Der Kernrezeptor retinoic acid-related orphan receptor gamma (RORγ) steuert die, von Entzündungen ausgelöste, sog. „Emergency myelopoiesis". Er hat auch eine Schlüsselfunktion im Glukose und Lipid-Haushalt und stimuliert die Expression von Genen des zirkadianen Rhythmus. Studien mit RORc-defizienten Mäusen haben im Zusammenhang mit fortgeschrittenen Krebserkrankungen gezeigt, dass RORC eine essentielle Rolle bei der Differenzierung und Expansion von MDSCs spielt.

Hypothese und Zielsetzung: Wir nehmen an, dass Entzündungsstress zur abnormen Expression von RORC und Genen des zirkadianen Rhythmus führt, was mit dem zirkadianen Rhythmus interferiert und dadurch zu SBS und metabolischen Erkrankungen sowie Gewichtsverlust führen kann. Das Ziel dieser Studie war es zu bestimmen, welche funktionelle Relevanz die Immunsystem-vermittelte Interferenz mit Genen des zirkadianen Rhythmus und RORC hat. All dies wurde im Zusammenhang mit experimenteller Kolitis untersucht.

Methoden: Akute Kolitis wurde chemisch durch Zugabe von Natriumsulfatdextran (NSD) zum Trinkwasser von WT C57BL/6 und RORC-/- Mäusen induziert. Die Schwere des Krankheitsverhaltenssyndroms wurde durch Messung von Bewegungsaktivität und Kerntemperatur unter Gebrauch von radio-telemetrischen intraperitonealen Sensoren bestimmt. Die Expression der Gene des zirkadianen Rhythmus im Dickdarm und in der myeloiden Zellpopulation der Milz wurde durch RT-qPCR quantifiziert. Die myeloide Zellpopulation und die Expression von RORC in der Milz und Dickdarm und Vorgängerzellen im Knochenmark wurden mittels Durchflusszytometrie bestimmt.

Ergebnisse: Wir beobachteten Ci-sCG im Dickdarm als auch in Monozyten und Neutrophilen der Milz von Mäusen mit NSD-induzierter Kolitis. Ci-sCG blieb auch bei Rückgang der Entzündung erhalten, was mit Zunahme des ursprünglichen Körpergewichts gekoppelt war. Interessanterweise zeigten jene Tiere, die einen schwereren Kolitisverlauf und Kachexie aufwiesen, eine Reversion von Ci-sCG. In diesen Tieren lagen die Expressionswerte von Genen des zirkadianen Rhythmus ähnlich hoch wie bei gesunden Kontrolltieren. Weiter beobachteten wir eine Expansion von Neutrophilen auf Kosten der entzündlichen Monozyten (beide Population wiesen hohe Expressionswerte für RORC auf) und einen Anstieg der RORChi Granulo-Monozytenvorgängerzellen (GMPs) im Knochenmark. RORC-defiziente Mäuse waren vor NSD-induzierter Kolitis weitgehend geschützt. Sie zeigten geringere klinische und histologische Zeichen der Kolitis, hatten keine Hochregulation von Genen des zirkadianen Rhythmus, welche in abgemagerten WT Mäusen beobachtet wurde und sie zeigten keine Entzündungs-vermittelte Emergency myelopoiesis".

Schlussfolgerung: Unsere Erkenntnisse weisen darauf hin, dass die zirkadiane Anpassung des Immunsystems bei Entzündungsstress für die Heilung von Entzündungen essentiell ist.

Schlüsselworte: Chronisch-entzündliche Darmerkrankungen, Kachexie, Gene des zirkadianen Rhythmus, Zytokine, RORC, Myelopoese

1.2 Summary (English)

Background and Aims: Immune activation in inflammatory diseases leads to cytokine mediated suppression of circadian clock gene expression (Ci-sCG), including *Clock* and *Period* genes and *Proline and acidic amino acid-rich basic leucine zipper (PAR bZip)* transcription factors *D-site albumin promoter-binding protein (dbp), tyrotroph embryonic factor (tef)*, and *hepatic leukemia factor (hlf)* in myeloid cells.
Cachexia is an energy-wasting syndrome driven by immunologic and metabolic imbalance, prevalent in the last stage of most chronic inflammatory disorders, including cancer. This disorder is characterized by severe body weight, fat and muscle loss, leading to fatigue, and increased susceptibility to secondary infections, recently proposed to be due to the presence of immature myeloid-derived-suppressor cells (MDSCs).
The nuclear receptor retinoic acid-related orphan receptor gamma (RORϒ) drives inflammation-triggered "emergency" myelopoiesis, has key functions in glucose and lipid metabolism and mediates the peak expression level of clock genes, without affecting their rhythmic expression. Importantly, studies with RORC deficient mice, in the context of advance cancer disease, have revealed a crucial role of RORC in promoting the differentiation and expansion of MDSCs.

Hypothesis and aims: We hypothesize that inflammatory stress, leading to abnormal clock gene and RORC expression might disrupt circadian rhythms contributing to Sickness behavior syndrome (SBS) and metabolic disorders, such as cachexia. The aim of this study was to determine the functional relevance of the immune mediated interference of clock gene and RORC expression, in the context of experimental colitis.

Methods: Acute colitis was induced in WT C57BL/6 and RORC-/- mice by dextran sulphate sodium added to the drinking water. SBS was assessed by monitoring locomotor activity and core body temperature using radio-telemetry intraperitoneal sensors. Clock gene expression was determined by RT-qPCR in colon, spleen and splenic myeloid populations. Myeloid subsets and RORC expression in spleen and colon, and bone marrow myeloid precursors were examined by flow cytometry.

Results: We report Ci-sCG in the colon and in splenic monocytes and neutrophils from mice with DSS induced colitis. Ci-sCG persisted during resolution of acute colitis and was associated with recovery of initial body weight. Interestingly, those animals, which developed a more severe colitis course and cachexia, had a reversion of the Ci-sCG, showing comparable clock gene expression levels to healthy controls. Furthermore, animals that failed to resolve DSS-induced inflammation showed an expansion of neutrophils at expense of inflammatory monocytes (both populations characterized by high RORC expression levels), as well as an increase in $RORC^{hi}$ granulo-monocyte precursors (GMPs) in the bone marrow. Importantly, RORC deficient mice were protected from DSS-associated cachexia, showing a

decreased clinical and histological score, lacked peak expression of clock genes observed in cachectic WT mice and showed a failure in inflammation-induced “emergency” myelopoiesis.

Conclusion: Our results suggest that circadian adaptation of immune and energy metabolism to inflammatory stress and increased demand might be crucial in resolution of inflammation and survival.

Key Words: Inflammatory bowel disease; clock genes; cachexia; RORC.

Chapter 2.

INTRODUCTION

Chapter 2. INTRODUCTION

2.1 Sickness behavior syndrome

Sickness behavior syndrome or SBS describes the collective behavioral and physiological adaptations of the host to inflammatory processes, such as viral or bacterial infections and sterile tissue injury. It comprises well-known symptoms, such as: fever, fatigue, anorexia, body weight (BW) loss, social withdrawal and depression. The psychological and behavioral components of SBS are mediated in the central nervous system (CNS) and represent, together with fever response and associated neuroendocrine changes, a highly organized strategy of the organism to fight inflammation and/or infection (Dantzer, 2001). This adaptive strategy of the organism is mediated by proinflammatory cytokines produced by activated cells of the innate immune system, and triggered by the contact of specific pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) with pattern recognition receptors (PRRs) and danger recognition receptors (DRRs), respectively. These cytokines include mainly interleukin (IL) 1 (IL-1α and IL-1β), IL-6, and tumor necrosis factor a (TNF-α), which can act on the CNS via two communication pathways: by the afferent neurons that innervate the inflamed tissue site or by a humoral pathway that involves the production of these cytokines by phagocytic cells in the circumventricular organs (CVOs) and choroid plexus (CP) in response to circulating PAMPs/DAMPs/cytokines, followed by the propagation of these immune signals into the CNS parenchyma (Konsman et al., 2002) **Fig. 1A**.

The action of pro-inflammatory cytokines in the CNS also mediates fever responses as well as the activation of the Hypothalamic-pituitary-adrenal (HPA) axis. Fever represents a regulated rise in body temperature resulting from thermogenesis and decreased thermolysis in response to an elevated set point for the regulation of body temperature. This set point is controlled by temperature-sensitive neurons in the preoptic hypothalamus and pyrogenic cytokines, such as IL-1β and IL-6, act in the CNS to induce fever (Romanovsky et al., 2005). Similarly, IL-1β acts in the paraventricular nucleus of the hypothalamus on corticotropin releasing hormone (CRH) producing neurons, leading to CRH release in the portal blood and the subsequent production of corticotropin from the pituitary, which, in turn, increases the release and secretion of glucocorticoids (GC) by the adrenal cortex (Dantzer, 2004).

Cytokines can act indirectly on the CNS by activating afferent vagal nerves, one of the major routes of visceral sensibility, which contain perineural macrophages (MPs) and dendritic cells (DCs) that can as well respond to inflammatory stimuli by their surface receptors (Goehler et al., 1999) **Fig. 1B**. Vagotomy experiments have confirmed the role of the vagus nerves in the transmission of information from the periphery to the CNS through the activation of the brainstem, hypothalamus (HP) and limbic structures, in response to peripherally administered lipopolysaccharide (LPS) or IL-1β. Accordingly, SBS was abrogated in vagotomized animals (Bluthe et al., 1994; Dantzer, 2004; Wan et al., 1994). However, while vagal

afferents are important for the behavioral component of SBS, their contribution in cytokine-induced fever and activation of the HPA axis is less relevant (Dantzer, 2004; Konsman et al., 2000).

Conversely, the action of DAMPS, PAMPs or circulating cytokines on macrophage-like cells in CVOs and brain endothelium results in the local production of cytokines and molecular intermediates, such as prostaglandins of the E2 series (PGE_2) and nitric oxide (NO). PGE_2 represent one of the main mediators of cytokine-induced fever and activation of the HPA axis (Rivest et al., 2000) diffusing into the brain parenchyma and acting on neuronal receptors in the brainstem and the hypothalamic neural structures involved in the control of the HPA axis activity and the regulation of body temperature (Dantzer, 2004; Lenczowski et al., 1999).

Of note, SBS can also occur in the absence of infection. For instance, high-mobility-group box1 (HMGB1) is an ubiquitous DNA-binding protein, which is released from the cells after sterile injury from hypoxia, cytotoxicity or physical damage to cell integrity (Tracey, 2010). Subsequently, HMGB1 is sensed by innate immune cells as an endogenous danger signal or DAMP, triggering signal transduction through PRRs and leading to their activation and the release of cytokines that mediate the neurological and metabolic manifestations of SBS, that are qualitatively similar regardless of whether the cytokine release was elicited by endogenous or exogenous stimulating agent (Tracey, 2010) **Fig. 1A.**

In mammals, the central clock in the suprachiasmatic nucleus (SCN) integrates light–dark cycle input and synchronizes the autonomous oscillators in peripheral tissues. The circadian clock regulates the rhythmic expression of downstream genes involved in behavior, reproductive and neuroendocrine functions, immunity and metabolism. Proinflammatory cytokines such as TNF-α and IL-1β have been shown to mediate clock-gene-dysregulation by inhibiting the binding of clock transcription factors (TFs) to E-box regulatory elements in clock gene promoters (Cavadini et al., 2007) (*See next section 2.2*). Importantly, the attenuation of clock genes has been suggested as a putative cause for the development of SBS in autoimmune, infectious and non-infections diseases, including sterile injury conditions like trauma or ischemia.

The proinflammatory cytokines that are produced by activated innate immune cells serve as sensory signals that are recognized and interpreted by the brain. The brain representation of peripheral immune activation resets the organism's priorities to enable the subjects at risk to deal with inflammatory processes in the most efficient way (Dantzer, 2004, 2006). However, in chronic activation of the innate immune system, SBS can become pathologic and can precipitate the development of depressive disorders leading to a poor quality of life (Dantzer, 2006).

As previously described, TNFα and IL1β, are two cytokines, among many others, involved in SBS development. In turn, they lead to down-regulation of clock genes, which has been suggested as an

important cause of SBS, in contrast to interferons (IFNs) and IL-6. Thus, this led to our interest of analyzing clock expression in myeloid cells, the main producers of these cytokines, *in* vivo in the context of inflammatory bowel disease and their implication to disease progression and outcome.

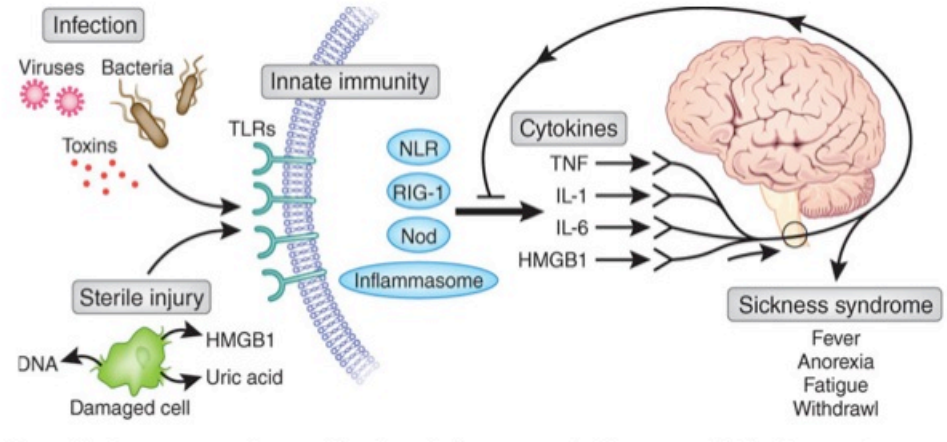


Figure 1A. Immune–neural connections: how the immune system's response to infectious agents influences behavior (McCusker R. et al. 2013)

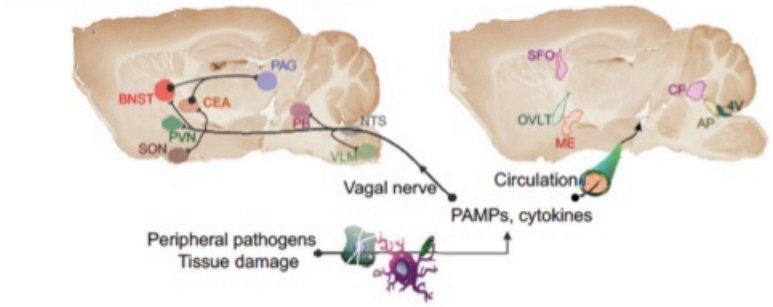


Figure 1B. Neuronal and humoral activation of the brain by the periphery (Dantzer et al. 2008).

2.2 The mammalian clock system.

Molecular and Cellular Basis of Circadian Clocks

The term circadian is derived from "circa" which means "approximately" and "dies" which means "day." It is known that obedience to temporal niches in all organisms is governed by a molecular circadian clock. These clocks are synchronized by the 24-h patterns of light and temperature produced by the earth's rotation. Circadian clocks are intrinsic, time-tracking systems that enable organisms to anticipate environmental changes (such as food availability and predatory pressure) and allow them to adapt their behavior and physiology to the appropriate time of the day (Schibler and Sassone-Corsi, 2002). Feeding behavior, sleep–wake cycles, hormonal levels, and body temperature are just a few examples of physiological circadian rhythms, with light being the principal zeitgeber ("time giver"; ZT). Other zeitgebers are feeding time and temperature (Brown and Azzi, 2013; Buhr and Takahashi, 2013).
Circadian rhythms regulate a wide array of metabolic and physiologic functions, like body temperature, blood pressure, circulating hormones, metabolism. Importantly, these rhythms persist in the absence of light–dark cycles and in many cases in the absence of sleep–wake cycles.
Accumulating epidemiological and genetic evidence indicates that disruption of circadian rhythms can be directly linked to many pathological conditions, including sleep disorders, depression, metabolic syndrome, and cancer. Intriguingly, a number of molecular gears constituting the clock machinery have been found to establish functional interplays with regulators of cellular metabolism and cell cycle (Buhr and Takahashi, 2013; Green et al., 2008).

The three fundamental parts of circadian clocks are the following: (1) an input pathway that includes detectors to receive environmental cues (or ZTs) and transmits them to the central oscillator; (2) a central oscillator that keeps circadian time and generates rhythm; (3) and output pathways through which the rhythms are manifested via control of various metabolic, physiological, and behavioral processes. Distinguishing characteristics of circadian clocks are being entrainable (synchronizable by external cues), self-sustained (oscillations can persist even in the absence of ZTs), and temperature compensated (moderate variations in ambient temperature does not affect the period of circadian oscillation) (Buhr and Takahashi, 2013).

The central pacemaker

In mammals, the SCN of the HP, is the master circadian clock, a "master synchronizer" for the entire body. Most tissues and cell types have been found to display circadian patterns of tissue-specific gene expression when isolated from the SCN, therefore, the SCN serves to synchronize the individual cells of the body to a

uniform internal time more like the conductor of an orchestra rather than the generator of the tempo themselves (Buhr and Takahashi, 2013). The mammalian SCN is entrained to light cycles in the environment exclusively by ocular photoreception, via the retinohypothalamic tract, and relays phase information to the rest of the brain and body via a combination of neural, humoral, and systemic signals, setting the phase of behavior and physiology throughout the body (Buhr and Takahashi, 2013) **Fig. 2**.

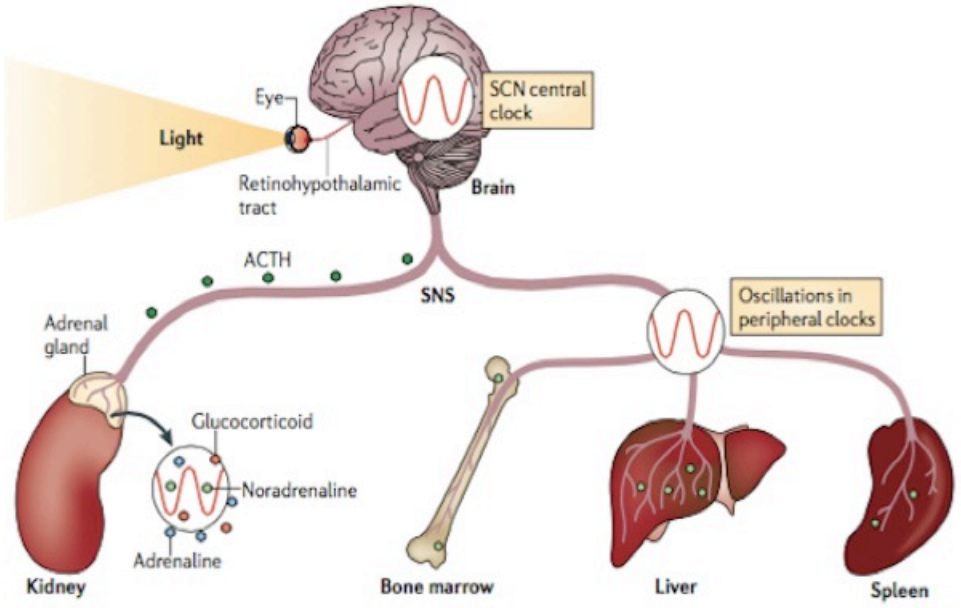


Figure 2. Entrainment and synchronization of the clock system. (Scheiermann et al. 2013)

Mechanism of the Molecular Circadian Clock

The molecular machinery that regulates circadian rhythms comprises of a set of genes, known as "clock" genes, whose products interact to generate and maintain circadian rhythms (Buhr and Takahashi, 2013; Sahar and Sassone-Corsi, 2013). The clockwork consists of an integral network of several interlocking positive and negative transcriptional and translational feedback loops (TTFLs) **Fig. 3**. Circadian locomotor output cycles kaput (CLOCK) and Aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL1 or BMAL1) are the positive regulators of the mammalian clock machinery, which regulate the expression of clock-controlled genes (CCGs) as well as the expression of negative regulators of the cycle: cryptochrome (CRY)1-2 and period (PER)1 -3 families. CLOCK and BMAL1 are TFs that heterodimerize through the PAS domain and induce the expression of CCGs by binding to their promoters at E-boxes [CACGTG]. Once a critical concentration of the PER and CRY proteins is accumulated, these proteins

translocate into the nucleus and form a complex to inhibit CLOCK-BMAL1-mediated transcription, thereby closing the negative feedback loop. In order to start a new transcriptional cycle, the CLOCK-BMAL1 complex needs to be derepressed through the proteolytic degradation of PER and CRY. Core clock genes (such as *Clock*, *Bmal1*, *Per*, *Cry*) are necessary for generation of circadian rhythms, whereas CCGs (i.e. Nampt) are regulated by the core clock genes. The CLOCK/BMAL1 dimers also initiate the transcription of a second feedback loop, which acts in coordination with the loop described above. This involves the E-box-mediated transcription of the orphan nuclear-receptor genes Retinoic acid-related orphan receptor (*Ror*) α-ϒ and nuclear receptor subfamily 1, group D, member 1-2 (*Nrd1-2* or *Rev-erb-α-β*). The REV-ERB and ROR proteins then compete for ROR elements (RORE) binding sites within the promoter of Bmal1, where ROR proteins initiate Bmal1 transcription and REV-ERB proteins inhibit it (Takeda et al., 2012). A separate set of PAR bZip genes, including the albumin D-box-binding protein (*Dbp*), tyrotroph embryonic factor (*tef*), hepatic leukemia factor (*hlf*) and nuclear factor interleukin 3 regulated (*Nfil3*) (Buhr and Takahashi, 2013), contain D-box elements in their promoters and make up another potential transcriptional loop. It has been proposed that the three known binding elements together provide the necessary delay to cycle at near 24 h: E-box in the morning, D-box in the day, and RORE elements in the evening (Minami et al., 2013).

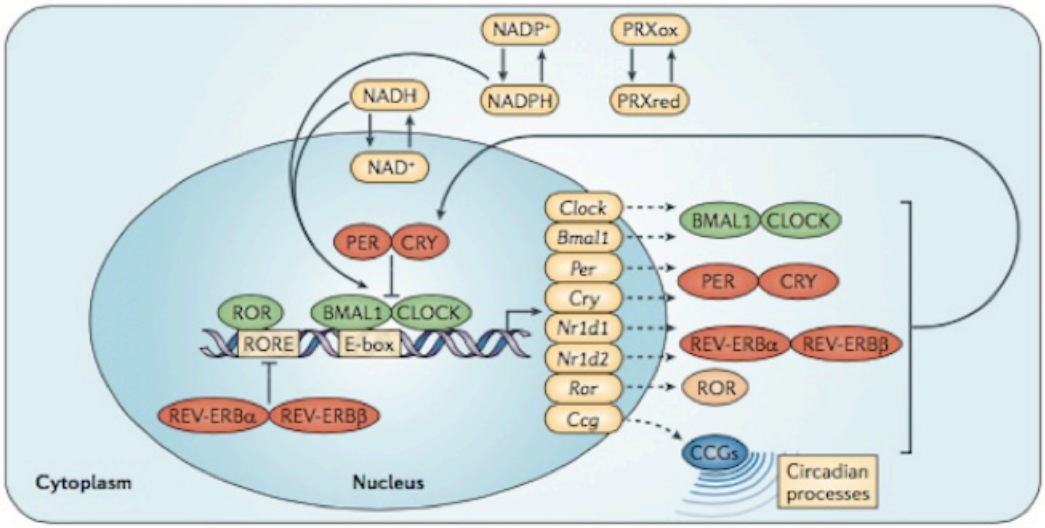


Figure 3. Molecular clockworks. (Scheiermann et al. 2013)

Non-transcriptional circadian rhythms

Precision within the circadian clock depends on nuclear TTFLs, but there is evidence that circadian, non-transcriptional, and non-translational cytosolic rhythms crosstalk with nuclear rhythms to maintain circadian timing. Therefore, this suggests that cellular metabolites may affect the transcriptional-translational canonical clock system and vice versa (Eckel-Mahan and Sassone-Corsi, 2013; Eckel-Mahan et al., 2012; O'Neill and Reddy, 2011; O'Neill et al., 2011).

Circadian rhythms which are independent of transcription have been discovered in higher organisms. For instance, in *Ostreococcus tauri* algae, transcription stops in the absence of light; however, the 24-h oxidation cycles of the antioxidant proteins, peroxiredoxins (PRXs), continue in constant darkness (O'Neill et al., 2011). Similarly, in human red blood cells, which lack nuclei, PRXs are oxidized (PRXred→ PRXox; **Fir. 3**) with a circadian rhythm (O'Neill and Reddy, 2011). These transcription-lacking-oscillators are also temperature entrainable. Importantly, however, in nucleated cells the transcriptional clock influences the cytoplasmic peroxiredoxin clock (O'Neill and Reddy, 2011).

Peripheral Clocks

The transcriptional feedback loop described above (**Fig. 3**) can be observed not only in the SCN but also in nearly every mammalian tissue (Brown and Azzi, 2013). At a single-cell level, the molecular clockwork of TTFLs can be observed as autonomous single-cell oscillators (Buhr and Takahashi, 2013). In addition to the core clock genes, hundreds or even thousands of genes are expressed in a circadian manner in various tissues. Approximately 10 % of the transcriptome displays robust circadian rhythmicity (Panda et al., 2002a). Circadian gene expression is tissue-specific and optimized to best accommodate the tissue's respective function throughout a circadian cycle (Buhr and Takahashi, 2013).

As mentioned above, light synchronizes the SCN to the external environment, and the SCN controls circadian fluctuations of body temperature. Physiologic fluctuations in temperature can entrain all peripheral oscillators that have been examined. This SCN output serves as an input to the circadian clocks of peripheral tissues whose outputs are the various physiological and transcriptional rhythms seen within the local cells throughout the body (Brown et al., 2002; Buhr and Takahashi, 2013) **Fig. 2**.

Epigenetic control of circadian rhythms

"Epigenetics" literally means "above genetics" and it is defined as the study of heritable changes in gene expression that does not involve any change to the DNA sequence. Such changes in gene expression

involve a variety of mechanisms including post-translational modifications of histones, remodeling of chromatin or methylation of DNA on CpG islands. Many of these epigenetic events are crucial in regulation of cellular metabolism and survival. Histone acetylation is a mark for activation of transcription, which is achieved by remodeling the chromatin to make it more accessible to the transcription machinery (Jenuwein and Allis, 2001; Sahar and Sassone-Corsi, 2013). Histone methylation, on the other hand, acts as a signal for recruitment of chromatin remodeling factors, which can either activate or repress transcription. DNA methylation leads to compaction of the chromatin and causes gene silencing. Genes encoding circadian clock proteins are regulated by epigenetic mechanisms, such as histone phosphorylation, acetylation, and methylation, which have been shown to follow circadian rhythm (Etchegaray et al., 2003; Masri and Sassone-Corsi, 2010; Ripperger and Schibler, 2006; Sahar and Sassone-Corsi, 2013).

Epigenetic control of the genome constitutes a critical interface of transducing signals, such as light or nutrient availability, and how these are interpreted by the cell to regulate gene expression, generating permissive or silenced states for transcription. CLOCK-BMAL1-mediated activation of CCGs is coupled to circadian changes in histone modification at their promoters. Chromatin modifiers, such as the deacetylase *Sirtuin 1* (SIRT1) and histone deacetylase 3 (HDAC3) or the methyltransferase *Mixed lineage leukemia 1* (MLL1), are recruited to the promoters of the CCGs in a circadian manner. Interestingly, the central element of the core clock machinery, the transcription factor CLOCK, also possesses histone acetyltransferase (HAT) activity. Of note, in the absence of these chromatin modifiers, rhythmic expression of the CCGs is abolished (Sahar and Sassone-Corsi, 2013) **Fig. 4**.
CLOCK-BMAL1-mediated activation of CCGs has been shown to be coupled to circadian changes in histone acetylation at their promoters (Etchegaray et al., 2003). Since CLOCK binds to E-box regions of DNA, the HAT activity of CLOCK can selectively remodel chromatin at the promoters of CCGs and is essential for circadian gene expression. The enzymatic activity of CLOCK also allows it to acetylate non-histone substrates such as its own binding partner, BMAL1 (Hirayama et al., 2007). CLOCK specifically acetylates BMAL1 at a conserved residue, an event that facilitates CRY-dependent repression. On the other hand, histone methylation is also important for circadian gene expression. (MLL1), a methyltransferase that methylates histone H3 at lysine 4 (H3K4), associates with CLOCK and is recruited to promoters of CCGs in a circadian manner, leading to transcriptional activation (Katada and Sassone-Corsi, 2010)

Intriguingly, the rhythmic deacetylation of histone H3 at the promoters of circadian genes is regulated by the deacetylase SIRT1, which is sensitive to Nicotinamide adenine dinucleotide $(NAD)^+$ levels (Asher et al., 2008). Interestingly, NAD^+/NADH ratio has been shown to regulate CLOCK/BMAL1's ability to bind DNA in vitro (Rutter et al., 2001). Since the NAD^+/NADH ratio is a direct measure of the energy status of a cell, the NAD^+ dependence of SIRT1 directly links cellular energy metabolism and deacetylation of target proteins (Sahar and Sassone-Corsi, 2013). Besides, circadian clock also control the expression of nicotinamide phosphoribosyl transferase (NAMPT), a key rate-limiting enzyme in the salvage pathway of

NAD^+ biosynthesis. The rhythmicity in the expression of this enzyme drives the oscillation in NAD^+ levels (Nakahata et al., 2009; Ramsey et al., 2009) **Fig.4**. Thus, cellular metabolism may play an important role in regulating the transcriptional state, and therefore the phase, of the clock (Sahar and Sassone-Corsi, 2013).

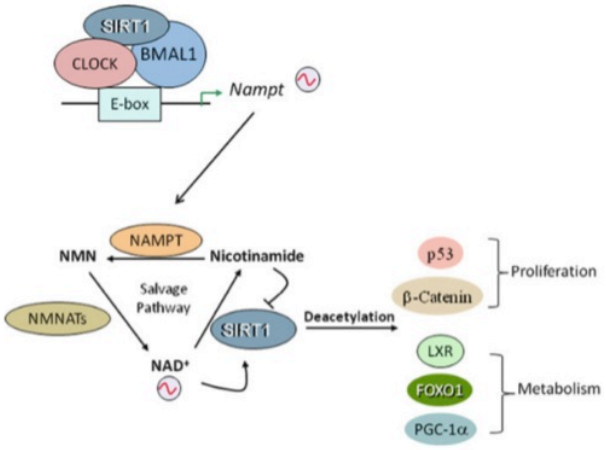


Figure 4. The epigenetic language of circadian clocks. (Sahar and Sassone-Corsi et al. 2013)

Clocks and cellular metabolism

In addition to sleeping-related disorders, circadian clocks are also directly linked with feeding and cellular metabolism, and several metabolic complications may result form a miscommunication with the circadian clock and metabolic pathways (Green et al., 2008). Many transcriptomes (McCarthy et al., 2007; Panda et al., 2002b; Storch et al., 2002; Vollmers et al., 2009; Zhang et al., 2014) and more recently, several proteomics (Mauvoisin et al., 2014; Reddy et al., 2006; Robles et al., 2014) and metabolomics (Adamovich et al., 2014; Ang et al., 2012; Dallmann et al., 2012; Eckel-Mahan et al., 2012; Minami et al., 2009) studies highlighted the pervasive circadian control of metabolism.

The rhythmic activation of selective pathways enables the organisms to optimize their ability to store and generate chemical energy, to minimize environmental stresses, and to reproduce through cell growth and division cycles. In mammalian tissues, major metabolic pathways exhibit robust diurnal rhythms, including glucose and lipid metabolism as well as mitochondrial fuel oxidation. Thus, this temporal organization of tissue metabolism is important for maintaining nutrient and energy homeostasis in mammals (Lin et al., 2008).

In order to adapt to the nutrient availability throughout light/dark cycles, the circadian clock controls as well the rhythmic activation of other cellular processes like autophagy, a conserved pathway activated in response to nutrient limitation, that results in the degradation of cytoplasmic components and the release of

nutrients to the circulation, providing a circadian steady supply of metabolites for energy homeostasis and anabolic pathways in the tissues. This process exhibits a robust circadian rhythm in the mouse liver and this regulation is mediated through CCAAT-enhancer-binding protein (c/EBP)-β. Importantly, as it will be described in more detail in next chapters, C-EBP- β is a major regulator of stress-induced-myelopoisis and adipogenesis, both high energy demanding processes (Ma et al., 2011).

On the other hand, mitochondria, as major suppliers of cellular energy through nutrient oxidation, encounters the permanent challenge of adapting to changes in nutrient supply and energy demand. Many metabolic diseases, such as diabetes and obesity are associated with a mitochondrial inability to deal with altered nutrient environment (Nunnari and Suomalainen, 2012). Neufeld-Cohen A. *et al.* described recently extensive oscillations in the mitochondrial proteome, which peak predominantly during the early light phase in mice (resting phase, RP). Remarkably, several rate-limiting mitochondrial enzymes that process different nutrients accumulate in a diurnal manner and are dependent on the clock proteins PER1/2. Notably, the diurnal regulation of mitochondrial respiration is dampened in mice lacking PER1/2 or in a high fat diet. The authors proposed that the circadian clock PERIOD proteins regulate the diurnal utilization of different nutrients by the mitochondria and thus, optimize mitochondrial function to daily changes in energy supply/demand. (Neufeld-Cohen et al., 2016).

Khapre R.V. *et al.* described an additional layer of circadian control of metabolism through the regulation of mammalian target of rapamycin complex 1 (mTORC1) activity, which is well known to act as a master switch between cell anabolic and catabolic programs (Khapre et al., 2014). The authors proposed that the circadian clock, through BMAL1-dependent mechanisms, inhibits mTOR signaling to actively suppress anabolism and prevent uncontrolled overuse of resources. Besides, suppression of mTOR activity has been shown to stimulate autophagy (Laplante and Sabatini, 2012). Therefore, circadian clock regulation of autophagy can also occur through mTORC1-dependent control. Anabolic processes like hematopoiesis, muscle growth, bone mineralization, epithelium and mucous cell renewal, synthesis of hormones and neurotransmitters, building up novel synaptic contacts etc. require significant amount of energy; therefore, these processes must be restricted under natural conditions where resources are limited, in order to maintain homeostasis and sustain basal metabolic rate. At the same time, excessive growth can prematurely deplete stem cell populations, resulting in accelerated aging resulting in poor fitness.
Furthermore, mTORC1-dependent metabolic reprogramming is a prerequisite for immune cell commitment and function (Donnelly et al., 2014; Katholnig et al., 2013; Soliman, 2013; Yang et al., 2014). Therefore, regulators of circadian mTOR signaling might be crucial for maintaining immune homeostasis to stress and demand.
Thus, the ability of the circadian clock to suppress anabolic processes through mTOR-dependent mechanisms can be evolutionary advantageous and important for survival of mammals. However, inflammatory conditions may trigger a dysregulation of the clockwork that may cause a permanent

activation of mTOR signaling pathways, leading to metabolic imbalance and altered immune homeostasis, affecting survival.

Circadian timekeeping system role in drug toxicity modulation

Gachon and colleagues showed the implication of PAR bZip CCGs in xenobiotic detoxification. They made use of PAR bZip triple KO mice, which age at an accelerated rate, and die prematurely. These mice appeared to be hypersensitive to xenobiotic compounds. Transcriptome analysis in murine liver and kidney revealed that PAR bZip proteins control the expression of many enzymes and regulators involved in detoxification and drug metabolism, such as cytochrome P450 enzymes, carboxylesterases, and constitutive androstane receptor (CAR), and that their deficiency in detoxification may contribute to their early aging (Gachon et al., 2006). Thus, these studies show for the first time the implication of clock genes in metabolic responses to stress and their impact in fitness.

In this thesis work we describe the development of cachexia in a model of acute colitis, characterized by increased BW loss and disease severity, which is linked to an altered clock gene profile, specifically in members of the network involved in drug detoxification and DNA damage response to stress. Surprisingly, the clock gene patterns observed in cachectic mice are different from mice which recover clinically from colitis and BW loss, after removal of the colitis inducing agent, in contrast to cachectic mice which fail to survive. This provides a novel link of the detoxification modulation role of the clock system in colitis pathogenesis, progression and outcome.

Clocks and cancer association

A number of epidemiological studies have linked defects in circadian rhythms to increased susceptibility to develop cancer and poor prognosis (Chen et al., 2005) and several studies in mice have linked clock genes and tumorigenesis (Gery et al., 2006). DNA repair processes and cell cycle checkpoints have been intimately linked with cancer due to their functions regulating genome stability and cell progression, respectively (Kastan and Bartek, 2004). The molecular mechanism of how circadian clock influences cancer development and progression can be explained by its regulation of cell cycle, DNA damage response, and cellular metabolism (Hunt and Sassone-Corsi, 2007; Sahar and Sassone-Corsi, 2013). Circadian clock proteins, such as PER1 and Timeless (TIM), interact with key checkpoint proteins (Gery et al., 2006) and circadian regulation of genes encoding key cell cycle regulators, such as Wee1 (G2 /M transition) (Matsuo et al., 2003), c-myc (G0 /G1 transition), and Cyclin D1 (G1 /S transition) (Fu et al., 2002) has been demonstrated in mammals.

Cellular DNA damage, either by intracellular agents (such as metabolic byproducts) or external agents (such as ionizing radiations), can cause cancer. However, cells have evolved several mechanisms to repair the damaged DNA. One of such repair mechanism is the nucleotide excision repair pathway. This pathway displays circadian oscillation in mouse CNS and peripheral tissues, possibly through oscillation in the

expression of the DNA damage-recognition protein xeroderma pigmentosum A (XPA) (Kang and Sancar, 2009). Thus, it is conceivable that uncoupling of this delicate balance could induce DNA damage, predisposing cells to tumorigenesis (Sahar and Sassone-Corsi, 2013).

Altered expression of clock components has been evidenced in animals models of inflammation and in human inflammatory diseases, such as inflammatory bowel disease (IBD), which will be described in detail in the next chapter. One of the complications derived from the chronicity and extent of colitis disease is colorectal cancer (CRC), which represents the third most commonly diagnosed malignancy and the fourth leading cause of cancer deaths (Breynaert et al., 2008; Sokol et al., 2008). Importantly, clock gene dysregulation, as well as genetic polymorphisms in several clock components, has been linked to the development of CRC and metastasis, evidencing again the role of chronodisruption in disease pathogenesis, cancer susceptibility and its progression (Huisman et al., 2015; Mostafaie et al., 2009; Sotak et al., 2013; Wang et al., 2011; Wood et al., 2008).

Circadian control of the immune system

Key parameters of the immune system in the blood exhibit circadian rhythms, most strikingly the number of circulating haematopoietic cells (HSCs), as well as hormonal and cytokine levels. Importantly, their oscillation in the circulation depends on the rest–activity phase of the species (Scheiermann et al., 2013). Of note, immune cellular and humoral components in the blood display opposite rhythms. Whereas immune cell numbers are released from the bone marrow (BM) at the onset of RP, thus having their acrophase in circulation during RP, levels of glucocorticoids, adrenaline, noradrenaline and the proinflammatory cytokines TNF-α and IL-1β peak during the onset of the active phase (AP). On the other hand, circadian leukocyte migration is regulated locally by sympathetic nerves and is mediated by the rhythmic expression of endothelial cell adhesion molecules and chemokines. Interestingly, adhesion molecule expression regulation is tissue-specific, suggesting that rhythms in the expression of these adhesion molecules drive the recruitment of haematopoietic cells to different tissues, which occurs preferentially during the AP. Accordingly, in mice, the homing of HSCs and neutrophils to the BM, as well as monocyte recruitment into tissue, peaks at night (AP). In the BM, the circadian homing of neutrophils may serve as a feedback mechanism that enables BM stromal cells to sense leukocyte concentrations in the blood and trigger the next round of cyclical haematopoietic cell release. In peripheral tissues, the circadian recruitment of leukocytes to tissues (peaking at the AP) may exist to replenish resident leukocyte populations and maintain the immunosurveillance of the body (Scheiermann et al., 2013). Thus, circadian regulation of hematopoietic cell trafficking in anticipation of the times of higher antigen exposure, which is potentially more likely to occur during the AP of the specie, might provide evolutionary advantage.

Nguyen et al. showed that circulating Ly6C^{hi} “inflammatory” monocytes but not “resident” monocytes, exhibit a diurnal oscillation that drives rhythmic migratory behavior. The authors selectively depleted Bmal1 in the myeloid lineage and observed that, remarkably, the oscillations ceased. A key observation

made by the authors was that BMAL1 expression in myeloid cells strongly suppresses inflammation. They showed that under steady-state conditions, BMAL1 binds rhythmically to the promoter regions of chemokine (C-C motif) ligand 2 (*Ccl2)* and *Ccl8*, genes that encode proinflammatory cytokines **Fig. 5**. Interestingly, however, the effect is anti-inflammatory because BMAL1 recruits the histone methyltransferase enhancer of zeste homolog 2 (EZH2) to these genes, resulting in epigenetic silencing of the loci. By contrast, in a lethal encounter with a pathogen like *L. monocytogenes* (or when BMAL1 is selectively depleted in the myeloid lineage), this inhibitory effect is ablated, resulting in increased systemic cytokine expression and dramatically reduced survival in response to infection. Thus, rhythmic BMAL1 repression of cytokines at peak sensitivity of the immune response is critical to downmodulate the inflammatory reaction (Nguyen et al., 2013).

All these facts (temporal increase in chemoattractants, leukocyte trafficking, proinflammatory cytokines and phagocytic ability in the hours approaching the start of AP are an indication of clock-controlled sensitivity and inmunosurvelliance ahead of activity and feeding, when the risk of infection would be higher. In short, the circadian system may partition the immune system into 2 states over the day: Firstly, a state of alert as the animal prepares to transition to activity, when the risk of infection/ injury is the greatest. This state would require increased leukocyte numbers along with increased sensitivity of inmune cells to infectious agents or danger signals. Secondly, a resting phase of the animal, giving the opportunity for resolution of inflammation and tissue repair (Druzd and Scheiermann, 2013; Nguyen et al., 2013).

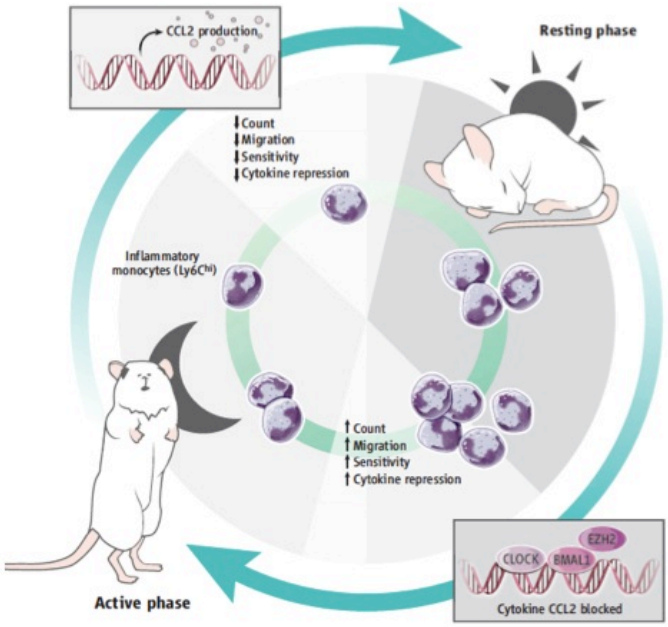


Figure 5. Some monocytes got rhythm. Druzd et al. 2013

2.3 Inflammatory bowel disease

IBD: definition, forms and prevalence.

The mammalian gastrointestinal tract (GIT) is continuously exposed to numerous bacteria as well as food-derived and environmental toxins and, as such, is highly vulnerable to disease.
Crohn's disease (CD) and ulcerative colitis (UC) are idiopathic, chronic inflammatory bowel diseases (IBD) empirically defined by clinical, pathological, endoscopic, and radiological features, and characterized by a dysregulated immunoinflammatory response to mucosal antigens presumably within the commensal gastrointestinal (GI) bacterial flora in genetically susceptible individuals. It is estimated that 4 per 1,000 Europeans and North Americans live with IBD. The onset of IBD typically occurs in young adulthood. Despite improved medical treatment options over recent years, the natural history of IBD appears largely unchanged and with a notable minority developing severe refractory disease activity or complications that ultimately requires surgery. IBD places a heavy burden on patients and society because it leads to life-long increased morbidity and disability including reduced health-related quality of life, reduced capacity for work and impaired productivity (Steenholdt, 2016).

SBS and IBD

Altering circadian rhythms significantly worsens the development of colitis in animal models, and preliminary human studies have shown that patients with IBD are at increased risk for altered sleep patterns. Alterations in circadian rhythmicity significantly might as well influence disease severity and risk of flare-up. Furthermore, in shift workers, disrupted sleep leads to an increased risk in a number of inflammatory and non-inflammatory GI diseases. In addition, altered sleep has the potential to modify the immune system and thus impact disease course in IBD (Swanson et al., 2011). Disrupted sleep may be one of the main causes of fatigue in IBD patients, independent of disease severity and with a major negative impact on their quality of life (van Langenberg and Gibson, 2010).

Intestinal inflammation

The GIT hosts a myriad of commensal bacteria that form a critical microbial metagenome assisting its host in nutrition (Zigmond et al., 2012). A single layer of epithelial cells, that separates the gut lumen from deeper tissues, ensures efficient nutrient uptake. This implies a very challenging scenario for the organism, as it has to tolerate the constant exposure to foreign commensal microorganims and their products, causing a low-grade chronic inflammation. Rather than remaining unresponsive, the host actively controls the microbiota composition by secretion of antimicrobial peptides and immunoglobulins (Peterson et al., 2007; Salzman, 2011). Conversely, defined intestinal commensals critically shape the gut-associated immune system (Atarashi et al., 2011; Ivanov et al., 2009). Importantly, the organism has to remain sensitive to

deviations from this "primed homeostasis" and rapidly respond to invading enteropathogens or injuries causing epithelial damage. Failure to maintain this fine balance and hyporresponsiveness in genetically predispose individuals results in chronic inflammatory bowel disorders such as CD and UC (Cho et al., 2008).

Mononuclear phagocyte compartment in the gut.

Monocytes are mononuclear phagocytes that represent key components of the innate immunity and provide a link with the adaptive immunity. In mice and humans, monocytes represent 4 and 10%, respectively, of the nucleated cells in the blood, with marginal pools in the spleen and lungs, that can be mobilized on demand. Monocyte development and survival depend on colony-stimulating factor 1 (CSF1 or M-CSF), showed by studies in CSF1 deficient mice, which exhibited severe monocytopenia (Ginhoux and Jung, 2014). Monocytes arise from myeloid precursor cells in primary lymphoid organs, including fetal liver and BM, during both embryonic and adult hematopoiesis, although in inflammatory conditions monocytes can also be produced in the spleen, as a well-known site for extramedular emergency myelopoiesis (Ginhoux and Jung, 2014). Monocytes originate from granolucyte-monocyte progenitors (GMPs) that will give rise to MP and DC precursors (MDPs) and subsequently to the recently identified common monocyte progenitor (cMoP), that will give rise uniquely to monocyte and their descendants but will not generate conventional (c) DCs or plasmacitoid (p) DCs (Ginhoux and Jung, 2014; Varol et al., 2007).

Two main subtypes of murine monocytes have been described in the circulation: Ly6C ^{hi}CX3CR1^{int} (classical monocytes), which are posed to traffic to sites of inflammation to supply peripheral resident mononuclear phagocyte subsets (Ajami et al., 2011; Geissmann et al., 2003; Ginhoux and Jung, 2014) and correlate with human CD14^{+}CD16^{+} cells (Cros et al., 2010) and Ly6C ^{lo} CX3CR1^{hi} ("patrolling monocytes") which correlate with human CD14^{lo} CD16^{+} and have been shown to adhere and crawl along the luminal vessel surfaces to survey endothelial integrity Moreover, these cells can respond to local danger signals and induce the recruitment of neutrophils, which upon activation will trigger focal endothelial necrosis and the resultant cell debris will be then cleared by Ly6C ^{lo} cells (Auffray et al., 2007). Accumulating evidence suggests that patrolling monocytes are derived from Ly6C ^{hi} cells differentiating in circulation to Ly6C ^{lo} cells (Varol et al., 2007) .

Notably, the intestinal MP compartment is unique in that it continuously recruits monocytes in homeostasis for its replenishment, probably due to the tonic low-grade inflammation caused by the microflora exposure of this tissue (Zigmond et al., 2012).

Ly6C ^{hi} monocytes seed the healthy intestinal LP to give rise to CD11c^{lo} tissue resident MPs (in a M-CSF dependent manner (Varol et al., 2009) that contribute to the maintenance of gut homeostasis. This population of non-migratory MPs is marked by prominent surface expression of F4/80 and the CX3CR1 chemokine receptor. Cx3CR1^{hi} MPs acquire in the gut, potentially under the influence of the microflora-exposed epithelium, a distinct non-inflammatory gene expression profile (Rivollier et al., 2012). CCR2

expression is essential to the recruitment of Ly6C^{hi} monocytes to the inflamed gut to become the dominant mononuclear cell type in the LP during acute colitis. In the inflammatory microenvironment, monocytes up-regulate PRRs, rendering them responsive to bacterial products to become pro-inflammatory effector cells, leading to the release of pro-inflammatory cytokines. With time, they will differentiate into migratory antigen-presenting cells (APCs, MPs and/or DCs) capable of priming naïve T cells giving rise to an adaptive immune response.

Tissue MPs, as scavenger cells, are continuously sampling their environment via receptor-mediated phagocytosis and present the processed antigens to T cells through major histocompatibility complex (MHC) Class II molecules. Upon contact with PAMPS/DAMPS, MPs activate, showing increased phagocytic and antigen-presenting capacity, as well as the production of proinflammatory cytokines and chemokines like TNF-α, IL-1β and CCL2. Through these mechanisms, MPs alert surrounding naive immune cells, and direct the influx of neutrophils and monocytes into the affected tissue. After antigen uptake, MPs migrate to secondary lymphoid organs to participate in the induction of the adaptive immune response. After pathogen/cell debris clearance, MPs undergo a phenotypic shift that results in the production of anti-inflammatory cytokines like IL-10 and transforming growth factor beta (TGF-β), leading to the resolution of inflammation (Mosser and Zhang, 2008; Zigmond et al., 2012).

Monocytes and MPs are major producers of TNF-α and IL-1β, representative cytokines that aggravates colitis. Notably, ablation of Ly6C ^{hi} monocytes has been shown to ameliorate acute gut inflammation (Zigmond et al., 2012). Similarly, pre-treatment of DSS-colitic mice with antibiotics specific for gram-positive bacteria, significantly reduced the severity of the colitis by selectively blocking the selective recruitment of monocytes/MPs (through epithelial downregulation of CCR2 ligands), but not of other myeloid cells (Nakanishi et al., 2015).

Aggravation of colitis is also seen in mice with a deletion of the IL-10 gene or IL-10 Rα in several models, including DSS and T-cell transfer colitis. Further, older IL-10−/− mice deficient in IL-10 develop spontaneous colitis with age. IL-10 has an essential role in maintaining GI homeostasis and genetic polymorphisms in IL-10 or IL-10 receptor (IL-10R) have been associated with IBD susceptibility. Moreover, pharmacologically administered IL-10 ameliorates colitis in mice by inhibiting intestinal inflammation and suppressing proinflammatory cytokine production, as signaling through the IL-10R downmodulates TNF-α production and other proinflammatory signaling through various mechanisms, including the induction of SOCS3. Noteworthy, it is known that the palliative actions of IL-10 in DSS-induced colitis predominantly results from its downregulation of NO and ROS production by LP MPs (Li et al., 2014).

Neutrophil role in intestinal inflammation

Polymorphonuclear leukocytes (PMN) dominate the initial leukocyte influx to sites of acute infection and inflammation. During intestinal inflammation, resident MPs contribute to the recruitment of neutrophils

through production of chemokines. Neutrophils present in the blood sense the chemoattractant gradient and traverse the vascular endothelium to reach the intestinal LP. As part of the normal gut inflammatory response, neutrophils are recruited to sites of infection or inflammatory stimuli within minutes, and the response peaks by 24–48 hours. Under certain physiological or pathological conditions, neutrophils cross the epithelium into the intestinal lumen. Upon reaching the inflammatory site, neutrophils selectively release monocyte chemoattractants, such as CAP18, cathepsin G, and azurocidin, leading to a second-wave of the inflammatory response. Interestingly, the depletion of intestinal mononuclear phagocytes (MPs and DCs, iMNP) has been reported to increase neutrophil infiltration and increase the severity of injury in the DSS-induced colitis model. However, when both neutrophils and iMNPs were depleted, colitis did not worsen, suggesting that neutrophils are deleterious in the absence of mononuclear cells during intestinal inflammation. Thus, intestinal mononuclear cells clearly have a role in regulating neutrophil infiltration during colitis (Fournier and Parkos, 2012; Qualls et al., 2006)

Crypt abscesses, which are a classic feature of intestinal infection or active IBD, result from transmigration of massive numbers of activated neutrophils across tight junctions and accumulation within colonic epithelial crypts. This process results in deformation of crypt architecture and disease symptoms (Fournier and Parkos, 2012) .

A major debilitating feature of IBD is the persistence of mucosal epithelial wounds/ulcers secondary to an excessive inflammatory response. During the acute inflammatory phase, PMN have a critical role in cleansing sites of injury by eliminating invading microbes. However, the cleansing process is associated with tissue injury. Afterwards, immune cells have a critical role in resolution events that restore tissue integrity. The resolution of inflammation requires that phagocytes exit along with efficient removal of both inflammatory mediators and cell debris in order to restore homeostasis. Although during the acute phase of inflammation PMN synthesize pro-inflammatory mediators, such as leukotrienes and prostaglandins, during resolution of inflammation their cellular biosynthetic pathway is switched to produce potent anti-inflammatory molecules such as lipoxin A4, which inhibits PMN recruitment and transepithelial migration (Fournier and Parkos, 2012). Indeed, PMNs have a crucial role during this particular phase of mucosal inflammation, as they contribute to the phagocytosis of cell debris accumulating at the wound site and participate in the biosynthesis of growth factors (GFs), such as vascular endothelial growth factor (VEGF), and pro-resolution mediators, such as lipoxins, resolvins and protectins, to facilitate wound healing (Fournier and Parkos, 2012).

On the other hand, the engulfment of apoptotic neutrophils cells by MPs, a process referred to as efferocytosis, is essential for maintenance of normal tissue homeostasis and a prerequisite for the resolution of inflammation. In addition, the removal of apoptotic neutrophils regulates granulopoiesis, and prevents secondary lysis and spillage of their noxious intracellular substances into circulation. Moreover, neutrophils

undergoing apoptosis trigger an anti-inflammatory and pro-resolving response in MPs, monocytes and DCs. Thus, apoptosis of neutrophils and its clearance by other phagocytes is a fundamental process required for tissue homeostasis and immunity, and its dysregulation can lead to exacerbated inflammation (Greenlee-Wacker, 2016).

The former paradigm that chronic inflammation was the result from overexpression and release of pro-inflammatory factors has been a matter of current debate, and increasing evidence supports the hypothesis that chronic inflammation occurs rather due to failure in the resolution process. Importantly, cachexia, a devastating wasting syndrome, common in many chronic inflammatory conditions, including IBD and cancer, has been suggested to develop from a failure in resolution of inflammation (Seelaender et al., 2012).

Experimental colitis

In the last few decades, numerous animal models have been developed to characterize the complexity of IBD pathogenesis, delineating underlying molecular mechanisms and evaluating potential human therapeutics (Chassaing and Darfeuille-Michaud, 2011). One of the most widely used mouse model of colitis employs dextran sodium sulfate (DSS), a chemical colitogen with anticoagulant properties, to induce disease, due to its rapidity, simplicity, reproducibility and controllability. DSS is a water-soluble, negatively charged sulfated polysaccharide with a highly variable molecular weight ranging from 5 to 1400 kDa. The mechanism by which DSS induces intestinal inflammation is unclear but is likely the result from epithelial damage in the large intestine, allowing the dissemination of luminal bacteria and their proinflammatory products into underlying tissue. Acute, chronic and relapsing models of intestinal inflammation can be achieved by modifying the concentration of DSS and the frequency of administration (Chassaing et al., 2014), allowing the investigator to study innate immune responses, depending on the duration of the DSS treatment. However, to study cellular characteristic adaptive immunity in mice, T cell transfer models are more commonly used.

2. 4. 'Emergency' myelopoiesis

Hierarchy of the hematopoietic system

The hematopoietic system is hierarchically organized with hematopoietic stem cells (HSCs) residing at the 'top', from which all cell types of the hematopoietic system are derived **Fig. 6**. HSCs is a rare population, representing only 0.01% of total BM cells, but that is highly enriched among Lin^- $Sca1^+$ c-Kit^{hi} cells, designated as LSKs (Kondo et al., 2003). Long term (LT)-HSCs are the only life-long self-renewing HSCs, which are enriched among $CD34^-$ LSK cells (Osawa et al., 1996). HSC divisions can result in self-renewal

or differentiation into multipotent progenitors (MPPs) that loose self-renewal capacity and will later differentiate into highly proliferative lineage-committed progenitor cells (HPCs), either common myelo-erythroid progenitors (CMPs) or common lymphoid progenitors (CLPs), both with absent or limited self-renewal capacity. CMPs will segregate into either megakaryocyte-erythroid progenitors (MEPs) or granulocyte-monocyte progenitors (GMPs) (Akashi et al., 2000b) .

'Emergency' hematopoiesis

HSCs give rise to all of the blood cell lineages that are required for the proper function of an organism throughout its lifetime. Given the postmitotic nature of the majority of mature haematopoietic cells, combined with their relatively short half-life (which has been reported to range from a few hours to a few days in the case of neutrophils), mature blood cells need to be constantly generated from upstream precursors. Of note, the haematopoietic system is capable of rapid adaptation to haematopoietic stress, such as bleeding or severe infection, by increasing cellular output several-fold above steady-state levels in order to meet the higher demand for the respective blood cell type. This process is referred as 'emergency' hematopoiesis (Manz and Boettcher, 2014).

During systemic infection and inflammation, an emergency myelopopoietic response is often elicited, and it is characterized by increased output of cells of the myeloid lineage from the BM hematopoietic stem and progenitor cells (HSPCs), and their mobilization, together mature neutrophils, to peripheral tissues. Similarly, hematopoiesis is shifted towards erythrocyte production during stresses like hemorrhage and anemia (Zhao and Baltimore, 2015).

HSPCs may respond to inflammation/ infection via several mechanisms. There is some evidence of the existence of a 'neutrostat' system able to sense the phagocytosis of apoptotic neutrophils consumed during an infection/inflammation and thus signal the BM to replenish the neutrophil pool accordingly (Bugl et al., 2013; Stark et al., 2005). HSPCs can also respond to inflammatory cytokines produced by various hematopoietic and non-hematopoietic factors, such as thrombopoietin, IL-3, stem cell factors, Fms related tyrosine kinase 3 (Flt3) ligand, interferons (IFNs), TNF-α, TGF-β, IL-6, granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF and macrophage (M)-CSF). Importantly, they are able to sense PAMPS/DAMPs directly through PRRs and DRRs, respectively. Finally, HSPCs can as well respond to paracrine signals from the stem cell niche. Stem cell niche cells, including osteoblasts, perivascular stromal cells, mesenchymal stem cells (MSCs) and endothelial cells are affected in various ways during an infection, which can influence proliferation, differentiation, and mobilization of HSPCs (Zhao and Baltimore, 2015).

Regulation of stress-induced hematopoiesis by toll-like receptor (TLR) signaling

Briefly, TLRs bind to conserved PAMPs, such as lipopolysaccharide (LPS) and can signal through either Myeloid differentiation primary response gene 88(MyD88)-dependent or TIR-domain-containing adapter-inducing interferon beta (TRIF)-dependent pathways, leading to activation of the nuclear factor kappa light chain enhancer of activated B cells (NF-k B) and/or the interferon regulatory factor (IRF) family of TFs (Kawai and Akira, 2010) **Fig. 6**.

Nagai et al. demonstrated the presence of PRR expression in HSPCs, specifically TLR2 and 4. It is known that their stimulation leads to Myd88-dependent myeloid differentiation in vitro (Nagai et al., 2006). Besides, many authors have shown that in vivo stimulation by LPS in mice results in increased HSC proliferation, decreased quiescence, decreased long-term repopulation ability and skewed myeloid differentiation (Esplin et al., 2011; Massberg et al., 2007; Megias et al., 2012; Takizawa et al., 2011). Human HSPCs under TLR stimulation differentiate preferentially into the myeloid lineage as well (De Luca et al., 2009). Nevertheless, Scumpia et al. demonstrated that stress-induced hematopoiesis did not depend on any single TLRs, cytokines or IFNs, by using different KO mice in the context of infection, suggesting that a tremendous redundancy has evolved in the mammalian hematopoietic system to sense and respond to bacterial infection (Scumpia et al., 2010).

The debate over whether cytokines can instruct lineage choice of uncommitted HSPCs versus simply promoting preferential proliferation and survival of stochastically committed progenitor cells was elegantly solved by Mossadegh-Keller an colleagues, that taking advantage of time-lapse single cell imaging and single cell gene expression analysis showed that M-CSF, but not G-CSF or GM-CSF, directly induced PU.1 expression in long-term (LT)-HSCs, and instructed them towards myeloid lineage, in the absence of proliferative or survival advantage, thus providing a strong evidence that cytokines can instruct lineage choice in uncommitted HSCs (Mossadegh-Keller et al., 2013).

'Emergency' granulopoiesis

In contrast to local inflammation, which is contained by regular innate effector immune cells, during severe systemic bacterial infection, 'emergency' granulopoiesis is triggered, and it is characterized by blood leukocytosis, neutrophilia, and the appearance of immature neutrophil precursor cells in the peripheral blood, which during physiological steady-state conditions are only present in the BM (Manz and Boettcher, 2014). Soon after migrating to the inflammation/infection site, most neutrophils will undergo cell death as a consequence of their antibacterial effector functions, such as phagocytosis, degranulation and the release of neutrophil extracellular traps (NETs). Failure to control an infection locally leads to systemic bacterial dissemination, resulting in further neutrophil 'consumption'. Thus, to counterbalance neutrophil depletion and to meet the enormous demand for neutrophils during infection, steady-state granulopoiesis is switched to emergency granulopoiesis, characterized by considerably enhanced *de novo* generation of neutrophils,

accelerated cellular turnover and the release of both immature and mature neutrophils from the BM into the peripheral blood (Manz and Boettcher, 2014).

Activation pathways of 'emergency' granulopoiesis.

There are direct and indirect mechanisms leading to the activation of emergency granulopoiesis. In both cases, the switch from steady-state to emergency granulopoiesis requires pathogen sensing through PRRs. Upon systemic dissemination of bacteria, pathogen sensing can occur either in peripheral tissues or in BM, as both haematopoietic and non-haematopoietic cells express the molecular sensing machinery. As a consequence, granulopoietic cytokines (such as G-CSF (or CSF3)) are released, either from the sensing cells themselves or by other cell types, and their signaling leads to indirect activation of emergency granulopoiesis. However, as previously mentioned, HPCs also express PRRs and can directly sense systemically spreading pathogens, resulting in enhanced proliferation and granulocytic differentiation both in an autocrine cytokine-dependent or independent manner. Thus, circulating HSPCs could potentially give rise to haematopoietic offspring directly at the site of the infection in order to support effector immunity and promote tissue repair at the inflammatory foci (Manz and Boettcher, 2014) .

Cytokine and growth factor main axes in granulopoiesis.

It is not surprising that multiple redundant cytokine and GF pathways are involved in the regulation of granulopoiesis, due to its fundamental importance in host defense and survival. One of the most studied pathways in this context is the G-CSF–G-CSF receptor (G-CSFR or CSFR3) axis. G-CSF-deficient or G-CSFR-deficient mice have a 70–90% reduction in circulating absolute neutrophil counts, due to a defect in proliferation and granulocytic differentiation, at the level of myeloid progenitors, as well as an enhanced apoptosis of mature neutrophils. This demonstrates the essential role of G-CSF in steady-state granulopoiesis. However, its role in the context of 'emergency' granulopoiesis is to date more controversial (Manz and Boettcher, 2014). Though, neutrophil progenitor homeostasis can to be regulated by other GFs like GM-CSF (CSF2), although this becomes apparent only if the haematopoietic system is systemically challenged. Nevertheless, neither G-CSF nor GM-CSF are absolutely essential for steady-state or emergency granulopoiesis, as mice that are deficient in both GFs, as well as mice lacking all three major myeloid GFs (G-CSF, GM-CSF and M-CSF (CSF1)), still produce neutrophils in steady-state conditions. Moreover, *Csf3-/-Csf2-/-Csf1-/-* mice are able to mount a granulopoietic response in a sterile model of peritonitis, which shows that even the combined lack of these GFs does not completely prevent granulopoiesis (Manz and Boettcher, 2014). Therefore, there might be additional key molecules acting upstream of these CSFs involved in both steady-state and 'emergency' granulopoiesis.

Of note, IL-6 has been as well identified as a factor contributing to the granulopoiesis process. Il-6-/- mice challenged with *C. albicans* showed impaired neutrophil responses. Furthermore, animals lacking G-CSF, GM-CSF and IL-6 had severe postnatal survival rates and *in vitro* granulopoiesis studies lacking G-CSF

and GM-CSF showed remarkably decrease in the process when IL-6 was also absent. Thus, suggesting that extrinsic GF-mediated pathways are important for both steady-state and emergency granulopoiesis (Manz and Boettcher, 2014).

Major signaling cascades and transcriptional networks involved in granulopoiesis.

Myeloid specification in early HPCs and subsequent granulocytic differentiation during steady-state conditions require the coordinated temporal activation and function of a large number of myeloid TFs. C/EBPα is a crucial TF for steady-state granulopoiesis, as shown in C/EBPα -/- mice, which completely lack neutrophil as a result of a differentiation block at the transition from MPPs to GMPs **Fig. 6**. Blocked neutrophil differentiation partly results from a lack of expression of the C/EBPα target gene *Csf3r,* which supports the importance of the G-CSF–G-CSFR signalling axis in granulopoiesis. Of note, C/EBPα simultaneously restricts excessive proliferation by inhibiting the expression of genes that promote cell cycle progression such as *c-myc*, and *cyclin-dependent kinase* (*cdk*) *2* and *4*. However, whereas C/EBPα is the master regulator of steady-state granulopoiesis, C/EBPβ is crucial for the regulation of 'emergency' granulopoiesis (Manz and Boettcher, 2014) **Fig 6**.

G-CSF-induced signal transduction occurs via Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathways that involve STAT1, 3 and 5. Importantly, STAT3 has been shown to be crucial for granulopoiesis directly promoting the expression of C/EBPβ. G-CSFR signaling through JAK leads to nuclear translocation of STAT3 (pSTAT3), which directly stimulates *c-myc* transcription, competing with C/EBPα, thereby leading to inhibition of the C/EBPα–mediated- transcriptional repression of *c-myc* expression. As a net result, the proliferative effect of C/EBPβ outweights the anti-proliferative effect of C/EBPα, resulting in enhanced myeloid progenitor cell proliferation and neutrophil generation (Manz and Boettcher, 2014). Given the competition between C/EBPα and C/EBPβ for binding to the *Myc* promoter, the reduced G-CSF levels that occur as a consequence of efficient pathogen clearance, and the resulting decrease induction of G-CSF-mediated-C/EBPβ expression, might be a mechanism for switching emergency granulopoiesis back to steady-state granulopoiesis (Manz and Boettcher, 2014).

Despite being the main positive pathway for emergency granulopoiesis, STAT3-mediated G-CSF-induced signaling can also activate suppressor of cytokine signalling 3 (SOCS3). SOCS proteins are negative feedback regulators of cytokine signaling. SOCS3 is recruited to tyrosine residues on G-CSFR, thereby inhibiting signal transduction through JAK–STAT3. SOCS3-deficient mice have prolonged STAT3 activation and are hyperresponsive to exogenous G-CSF administration, which leads to excessive neutrophilia, increased HSPC mobilization and splenomegaly (Manz and Boettcher, 2014). Similarly, it has been shown that G-CSF stimulation induces expression of the inhibitor of nuclear factor-κB (IκB) family member B cell lymphoma 3 protein (BCL-3) in a STAT3-dependent manner. BCL-3 inhibits the expression of TNF-α by MPs, and also regulates negatively TLR signaling. Importantly, although BCL-3 has been identified as a proto-oncogene, it is required to limit myeloid progenitor proliferation and differentiation

into neutrophils in an NF-κB-p50-dependent manner, thereby restrains excessive emergency granulopoiesis (Manz and Boettcher, 2014).

Importantly, Strauss and colleagues recently identified the nuclear receptor RORC as a novel regulator of 'emergency' myelopoiesis in response to the colony stimulating factors (CSFs) G-CSF and GM-CSF by promoting C/EBPβand suppressing *Socs3* and *Bcl-3* expression, in the context of cancer inflammation. The authors showed that RORC promotes the differentiation and expansion of myeloid-derived-suppressor cells (MDSCs) by a blockage in early hematopoietic progenitors, as well as the differentiation of M2 tumor-associated-macrophages (TAMs).

Role of HSPCs in intestinal inflammation

Powrie and colleagues provided evidence that HSC activity is dramatically modified during intestinal inflammation, with a marked increase in HSC proliferation and their accumulation not only in the BM but also at extramedullary sites of colitic mice. This was accompanied by a substantial skewing of HSC differentiation toward GMPs, which were increased in the BM as well as the inflamed spleen and colon. IFN-ϒ and GM-CSF promoted dysregulated hematopoiesis, acting at distinct points in stem and progenitor cell differentiation. Finally, the authors reported a direct link of GMPs to disease pathogenesis, showing aggravation of colitis following adoptive transfer of splenic GMPs (Griseri et al., 2012).

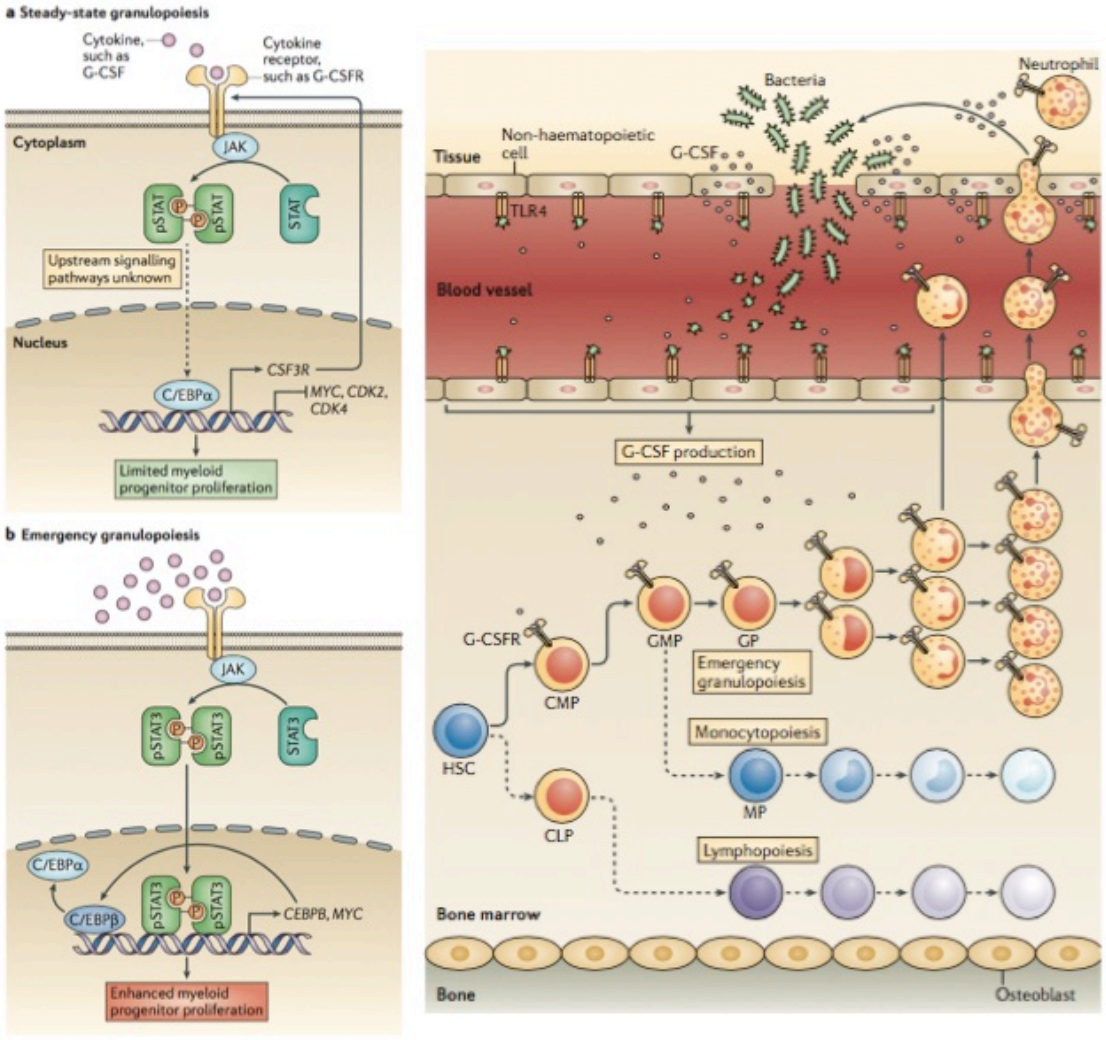


Figure 6. An integrated model of emergency myelopoiesis (Manz M. et al. 2014)

2.5 CACHEXIA

Cachexia definition

Cachexia is a metabolic syndrome resulting in overall weight, fat and muscle loss (Homsi and Luong, 2007). Etymologically, cachexia is a term originating from the Greek, *kakos* and *hexia*, meaning 'bad condition'. Although in the last few years several definitions have appeared, a certain consensus indicates that they all share two common factors: weight loss mainly from skeletal muscle and body fat and inflammation (Argiles et al., 2014). Cachexia accompanies many infectious and chronic inflammatory diseases, including cancer. The incidence of the syndrome among cancer patients is very high and it is well accepted that cachexia is indirectly responsible for the death of at least 20% of all cancer patients (Fearon

et al., 2011). Just to show some numbers, the prevalence in patients with colorectal and lung cancer is 60%, 80% in gastric, pancreatic, aesophageal and 70% head and neck cancer (Argiles et al., 2014).

Previously, cachexia was defined as a body mass index (BMI) of < 20 and/or an unintentional BW loss of ≥ 5% in the previous 6 months (over 12 months in the setting of underlying disease, such as cancer) (Maltoni et al., 1999). A more recent definition has been proposed, which would incorporate the ≥ 5% BW loss over 12 months or less, plus three of the following features: decreased muscle strength, BMI of <20, fatigue, anorexia, low fat-free mass index, and abnormal biochemistry (increased inflammatory markers such as CRP or IL-6, anemia (hemoglobin of <12g/dL), or low serum albumin of <3.2 f/dL) (Evans et al., 2008). Lean tissue wasting is only one component of the cancer cachexia response, which also includes fatigue, asthenia, anorexia, anemia, insulin resistance, protein catabolism, lipolysis, an hepatic acute phase protein response and increased susceptibility to secondary infections.

Cachexia etiology

Cancer cachexia is associated to poor prognosis and survival. Its aetiology is multifactorial and very complex, comprising changes in lipid metabolism and overproduction of inflammatory mediators, including cytokines produced by inappropriate activation of innate immunity.

In the recent past, cachexia was accepted as a chronic inflammatory state characterized by increased lipolysis, total oxidation of FAs, hyperlipidaemia, hypertriglyceridaemia, and hypercholesterolaemia, thought to be cause, partly, by increased inflammatory mediators in white adipose tissue or WAT (mainly TNF-α, which originally has also been termed cachexin) with the potential of altering homeostasis of the tissue (Batista et al., 2012). However, recently Fukawa and colleagues revealed that alteration in FA metabolism itself, as the first trigger of cachexia (Fukawa et al., 2016). Thus chronic inflammatory status may induce metabolic dysbalance in WAT and other tissues such as liver, characterized by increased mitochondrial FA oxidation and subsequently liberation of TAG and FAs, leading to ROS-induced-cellular stress and DNA damage that can ultimately impair muscle growth and cause muscle wasting.

On the other hand, recent evidence has linked cancer cachexia with the presence of an immature myeloid cell population that expands dramatically in the tumors and secondary lymphoid organs of animals with active tumor growth. These immature GR-1^+ $CD11b^+$ cells are metabolically active and secrete large quantities of inflammatory cytokines and chemokines, and their expansion is temporally associated with the development of cachexia (Winfield et al., 2008).

Importantly, RORC has a role in regulation and rhythmic expression of genes involved in lipid metabolism and as a NR, acts as a ligand-dependent TF able to sensor of FAs and hydroxycholesterol (Jin et al., 2010; Solt et al., 2011). In addition, novel RORC promotes the differentiation and expansion of immature myeloid derived suppressor cells (MDSCs) in advance cancer disease (Strauss et al., 2015b) **Fig. 7**.

2.6 MYELOID-DERIVED SUPPRESSOR CELLS (MDCSS)

Tumor growth is often associated with the aberrant systemic accumulation of MDSCs, which are a heterogenous population of cells composed of PMN neutrophils, monocytes, MPs, DCs and early myeloid precursors. MDSCs are well-known for their ability to inhibit T-cell proliferation and anti-tumor immune responses (Nagaraj et al., 2013). The best described mechanisms/mediators related to immunosuppression by MDSCs include expression of Arginase 1 (Arg1), nitric oxide (NO), peroxynitrite ($ONOO^-$) and reactive oxygen species (ROS); deprivation of L-cysteine (L-cys), essential aminocid required for T cell activation and function (Srivastava et al., 2010); up-regulation of cyclooxygenase 2 (COX) and prostaglandin E2 (PGE2) (Rodriguez et al., 2006); secretion of TGF-β(Filipazzi et al., 2012; Yang et al., 2008a) and induction of T regs (Nagaraj et al., 2013). Accumulation of MDSCs in these target tissues is a dynamic process associated with medullary and extramedullary myelopoiesis and subsequent cellular migration (Ueha et al., 2011).

MDSCs and resolution of inflammation

MDSCs have an important role exercising balance between host-defense-associated inflammation mounted during an immune response and inflammation-mediated tissue pathology (Ortega-Gomez et al., 2013). High dose of bacterial components such as LPS in circulation due to the DSS-induced-breakage of the mucosal intestinal barrier, could be a stimulus for MDSCs development, as it has been described by others that high dose of LPS infusion, as well as gram negative bacterial infection in the lung, can trigger a potent MDSCs expansion (Ray et al., 2013). More specifically, TLR4 activation by high LPS dose on Lin- precursor cells induces the production GM-CSF and IL-6, causing STAT5 and STAT3 activation, which can support the development and suppressive function of MDSCs, respectively (Arora et al., 2010; Chalmin et al., 2010; Condamine and Gabrilovich, 2011). Noteworthy, MDSC –mediated immunosuppression can be as well promoted by DAMPs such as S100A and heat shock proteins (Bunt et al., 2009; Cheng et al., 2008). This suggests that collateral tissue damage associated to the massive neutrophil recruitment to infected tissues due to a defective host ability of mounting an anti-inflammatory response that limits neutrophil infiltration, once the pathogen is cleared, as well as impaired elimination of apoptotic neutrophils, might lead to the release of DAMPs and the promotion of MDSCs differentiation and their suppressive capacity.

MDSCs and cachexia

Under certain pathological conditions, myeloid cell homeostasis is altered and immature forms of myeloid cells appear in tissues at the expense of protective mature effector populations. Murine PMN and M-MDSCs have been associated with immunosuppression in cancer and more recently, in different models of

infection (Arora et al., 2010; Tam et al., 2014), leading to tumor progression and pathogen dissemination, respectively. Importantly, recent evidence has posed the expansion of MDSCs as a trigger of cancer-cachexia, leading to altered host energy metabolism and eventually reduced survival to polymicrobial sepsis and endotoxemia. (Cuenca et al., 2014)

Sepsis and MDSCs

Acute tissue foci of inflammation, whether generated in response to infection, injury, noxious agents or autoimmunity, present a unique and challenging microenvironment: Hypoxia (low oxygen) or anoxia (complete lack of oxygen), hypoglycaemia (low blood glucose), acidosis (high H+ concentration) and abundant free ROS, along with the influx of specialized myeloid cells such as neutrophils and macrophages (Nizet and Johnson, 2009). These short-lived cells are rapidly mobilized in response to any change in tissue integrity or entry of pathogenic microorganisms. Quantitative deficiencies in the numbers of these specialized phagocytic cell types (for example, following cancer chemotherapy) or inherited defects in their core effector functions (for example, in chronic granulomatous disease) greatly increase susceptibility to recurrent or severe infections (Nizet and Johnson, 2009; Shalova et al., 2015). Monocytes and macrophages are believed to play an important role in orchestrating the host immune response during sepsis. These innate immune cells can potentially participate in both phases of sepsis, contributing to the ‘‘cytokine storm’’ and ultimately adopting an immunosuppressive phenotype whereupon they are unable to respond to secondary infections (Shalova et al., 2015).

Recently, Shalova and colleagues shed light on the molecular and cellular basis of human sepsis progression, demonstrating that blood monocytes from Gram-negative septic patients displayed functional plasticity, transiting from a pro-inflammatory to an immunosuppressive state during sepsis, and identified hypoxia inducible factor-1α (HIF-1α) as key mediator of monocyte re-programming under these conditions (Shalova et al., 2015). Furthermore, the authors showed that monocytes in sepsis exhibit hallmarks of endotoxin tolerance in vivo, characterized of defective cytokine release in response to LPS, impaired APC function (low expression of MHC-II and co-stimulatory molecules as well as reduced ability to induce T cell proliferation *ex vivo*) and increased expression of anti-microbial genes and higher phagocytosis ability (Shalova et al., 2015). HIF-1α regulates mammalian cell response to hypoxia and is induced by gram negative endotoxin challenge in mice (Rius et al., 2008). The drastic reduction in oxygen tension during inflammation (2.5–9% in healthy normoxic tissue versus oxygen versus <1% oxygen) imply that phagocytic cells must be adapted to generate energy and function effectively in oxygen-deprived conditions, especially as many common bacterial pathogens proliferate readily in anaerobic microenvironments (Nizet and Johnson, 2009). The HIF1α-deficient macrophages and neutrophils had reduced levels of cellular ATP (15–40% of WT levels), highlighting the crucial role of the TF for energy generation through glycolysis in these immune cells.

Besides, It is known that hypoxia upregulates IRAKM (or IRAK3), one of the few conserved negative regulators of TLR signaling pathway in mice and humans which has been implicated in endotoxin tolerance

(Fang et al., 2009; Kobayashi et al., 2002). Accordingly, its up-regulation has been demonstrated in human sepsis monocytes *in vivo,* being consistent with their impaired endotoxin tolerant phenotype (Shalova et al., 2015).

Intestinal microflora stimulates the colitogenic immune system through TLR signaling and its negative regulation is essential in maintaining intestinal homeostasis (Biswas et al., 2011). It has been described that *Irak-m* is closely associated with the presence of intestinal microbiota, as its deficiency enhances the production of pro-inflammatory cytokines in macrophages and intensifies experimentally-induced DSS colitis (Klimesova et al., 2013). Interestingly, the expression of IRAK-M is dependent on intestinal commensal flora, as its expression was reduced in mice re-derived into a germ-free environment, and introduction of commensal bacteria into germ-free mice induced IRAK-M expression. IL-10-/-IRAK-M-/- mice exhibited exacerbated colitis with increased inflammatory cytokine gene expression. However, we observed higher IRAKM expression in cachectic mice, which showed increased epithelial damage and leukocyte infiltration, as well as more severe disease course after DSS exposure.

2.6 NUCLEAR RECEPTORS:

The nuclear receptor (NR) superfamily is one of the primary classes of therapeutic drug targets for human disease, among those are the anti-inflammatory glucocorticoids, steroidal contraceptives and hormone replacement therapies ear (Kojetin and Burris, 2014). NRs are generally classified as ligand-regulated TFs. The binding of ligands to a region of the receptor called ligand-binding-domain (LBD) causes a conformational change which will result in a cascade of downstream events. Subsequent to ligand binding, the conformational change in the receptor facilitates the recruitment of transcriptional co-regulatory proteins to receptor-specific gene promoter complexes to activate or repress transcription (Kojetin and Burris, 2014). Typically, NR ligands are small hydrophobic molecules including steroid hormones, FAs, and lipophilic vitamin derivatives (Solt and Burris, 2012).

RORs, consisting of RORα-γ(a-c) are involved in many physiological processes, including regulation of metabolism, development and immunity, as well as circadian rhythms. Of note, there is a considerable overlap in the DNA response elements that are recognized by RORs and another family of NRs, REV-ERBs (α-β). Besides, both receptors are often co-expressed in the same tissues. Whereas RORs constitutively activate transcription, REV-ERBs activity is crucial for the dynamic regulation of target genes containing the DNA response elements (ROREs) that are responsive to both classes of NRs. Therefore, owing to the substantial overlap in expression patterns as well as in gene targets, REV-ERBs and RORs are often involved in the regulation of similar physiological processes (Kojetin and Burris, 2014).

Retinoic-acid-related orphan receptor gamma (ROR-γ, RORC)

RORC is a key regulator of cell differentiation, immunity, peripheral circadian rhythm as well as lipid, steroid, xenobiotics and glucose metabolism. The *Rorc* gene encodes two different protein isoforms: full length protein (RORC1) and the RORCγt splice variant (RORC2) (Ivanov et al., 2006). RORC binds DNA as a monomer to ROR response elements (RORE) containing a single core motif 5'-AGGTCA-3' preceded by a short A-T-rich sequence.

RORC regulates the circadian expression of clock genes such as *Cry1*, *Bmal1* and *Rev-erb-α* in peripheral tissues and in a tissue-selective manner (Takeda et al., 2012). It competes with REV-ERB-α for binding to their shared DNA response element (RORE) on the promoter of some clock genes such as *Bmal1*, *cry1* and *Rev-erb-α* itself, resulting in REV-ERB-α-mediated repression or RORC-mediated activation of the expression, leading to the circadian pattern of clock genes expression. Furthermore, it has been shown to influence the period length and stability of the clock (Takeda et al., 2012).

In addition, RORC is involved in the regulation of the rhythmic expression of genes involved in glucose and lipid metabolism. Of note, RORC is a key regulator of adipogenesis and controls adipocyte size and insulin sensitivity modulation in obesity (Meissburger et al., 2011). In the liver has specific and redundant functions with ROR-α, as positive or negative modulator of expression of genes involved in the metabolism of lipids, steroids and xenobiotics (Meissburger et al., 2011). Furthermore, it plays also a role in the regulation of hepatocyte glucose metabolism through the regulation of Glucose-6-phospahte (G6PC) and Phosphoenolpyruvate carboxykinase 1 (PCK1), key regulatory enzymes in hepatic gluconeogenesis (Solt et al., 2015).

On the other hand, the isoform 2 (RORC2) is essential for thymopoiesis and the development of several secondary lymphoid tissues, including lymph nodes and Peyer's patches. It is as well required for the generation of LTi (lymphoid tissue inducer) cells (Eberl et al., 2004; Villey et al., 1999; Xie et al., 2005).

In addition, RORC2 plays a key role; downstream of IL-6 and TGF-β and synergistically with ROR-α, for lineage specification of uncommitted CD4+ T-helper (Th) cells Th17 cells, antagonizing the Th1 program (Ivanov et al., 2006; Yang et al., 2008b).

Role of RORC in adipogenesis

Obesity is a major risk factor for developing type 2 diabetes and metabolic syndrome. During obesity, adipose tissue increases via hyperplasia (increase in cell number) through *de* novo differentiation of pre-adipocytes and/or hypertrophy (increasing cell size), and the balance between these two processes determines the metabolic outcome of obesity. Hypertrophic cells are characterized of being insulin resistant and they change their secretory profile towards pro-inflammatory adipocytokines , resulting in free FAs release, and cytotoxic FA accumulation in extra-adipose tissue. Of note, RORC is a negative regulator of adipocyte differentiation. Obese RORc-/- mice shows decreased adipocyte size, that are insulin sensitive,

leading to improved control of circulating FAs, and protection from hyperglycemia and insulin resistance (Meissburger et al., 2011). RORC interferes with the adipogenesis process by decreasing the expression of key adipogenic proteins, C/EBP-α, C/EBP-β, C/EBP-δ and peroxisome proliferator-activated receptor (PPAR)-ϒ, a key regulator of fatty acid storage and glucose metabolism (Meissburger et al., 2011). Thus, RORC could be involved in the pathogenesis of cachexia, considering its role in lipid metabolism and its ability as a NR to sense free FA and oxidized cholesterol (Jin et al., 2010), recently described as triggering cause of muscle wasting in cachexia (McCarthy, 2014)

Noteworthy, cholesterol efflux and circulating FAs have been showed to mediate myelopoisis, as well as lineage commitment and function of immune cells, including myeloid cells (Murphy et al., 2014). Recent studies suggest that disruption in cholesterol homeostasis or prolonged exposure to a hypercholesterolemic environment can mediate HSPCs lineage commitment and function of innate immune cells, including myeloid cells with skewed differentiation to monocytes and promote their polarization to M1-like macrophages once they enter the atherosclerotic lesion (Murphy et al., 2014).

Role of RORC1 in stress-induce myelopoiesis

As stated above, RORC1 has been recently proposed to orchestrate tumor-driven 'emergency' myelopoiesis, promoting the generation of MDSCs and TAM differentiation in response to CSFs, leading to tumor progression and increased metastasis (Strauss et al., 2015b). RORC effects in the hematopoietic compartment are proposed to be mediated by stimulating positive (C/EBPβ) and suppressing negative (SOCS3 and BCL3) regulators of granulopoiesis, as well as transcriptional mediators of myeloid progenitor commitment and differentiation to the monocytic/macrophage linage (IRF8 and PU.1). The authors demonstrated that RORC1 supported tumor-promoting innate immunity by protecting MDSCs from apoptosis, mediating M2 polarization of TAMs, as well as limiting tumor infiltration by mature neutrophils **Fig. 7**. Further, the authors showed that RORC expression was induced in myeloid cells by TLR4 stimulation, as well as by CSF1-3 and IL-1β stimulation. Of relevance, specific depletion of RORC1 in the hematopoietic compartment prevented cancer-driven granulo-monocytopoiesis, resulting in the inhibition of tumor growth and metastasis. Importantly, the $RORC^{+}$ MDSC accumulation was a hallmark of advance cancer disease and correlated with increased BW loss, tumor volume and metastasis number (Strauss et al., 2015b). Notably, cachexia is currently accepted as a chronic inflammatory and metabolic dysbalance that occurs at the end stage of most chronic inflammatory diseases, including cancer.

Therefore, regulation of 'emergency' myelopoiesis by nuclear TFs such RORC, that may adapt inflammatory stress responses to metabolic oscillations, suggests a novel molecular mechanisms that connects inflammation to metabolism.

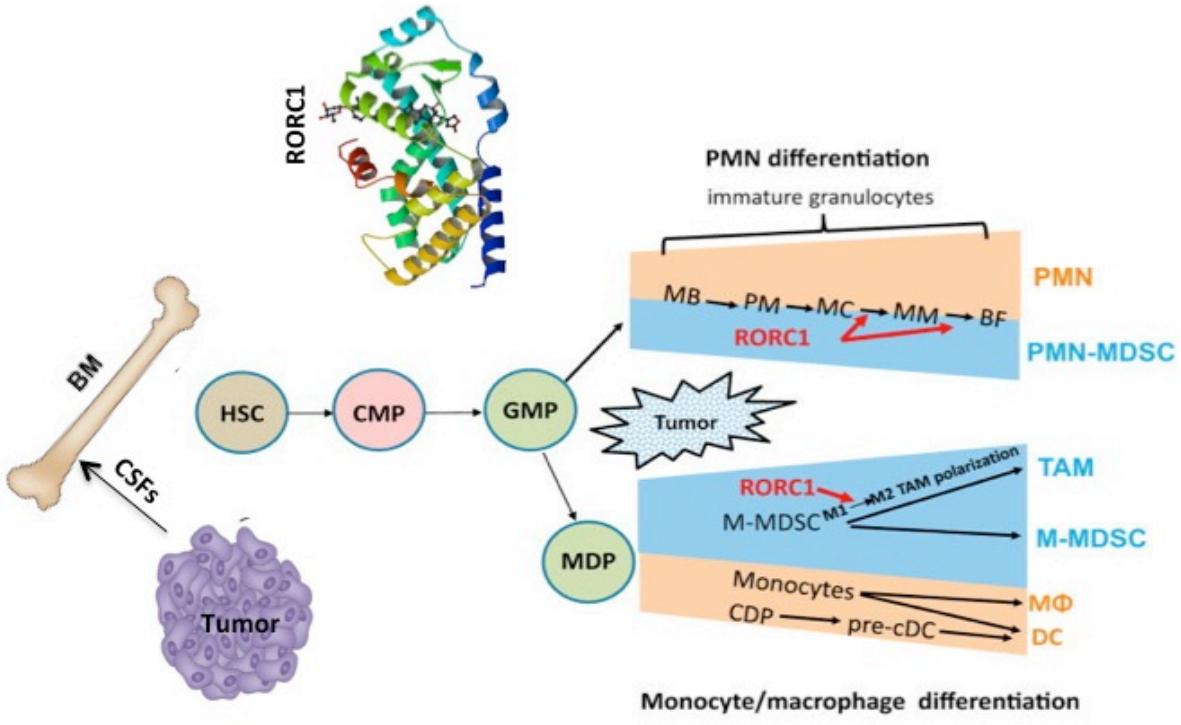


Figure 7. RORC1 Involvement in Myeloid Lineage Differentiation (Adapted from Strauss et al. 2015 and Gabrilovich D. et al. 2015)

2.7. THESIS AIMS AND HYPOTHESIS

Dampened circadian rhythms have the potential to modify the immune response and influence the disease course and outcome in GI inflammatory disorders like IBD, as it has been shown previously in animal models of colitis, and in human IBD patients. In addition, fatigue has been posed as one of the main factors with an impact in the quality of life of IBD patients, regardless of disease severity. Importantly, fatigue is one of the main components of SBS and cytokine-induced-clock gene dysregulation has been described to be a SBS determinant. Innate myeloid cells, as major producers of pro-inflammatory cytokines, namely TNF-α and IL-1β, might have a role in SBS development in the context of intestinal inflammation. They have been described to alter the expression of clock genes *in vitro* and *in vivo*, upon injection, in mice. Thus, as a first aim we are interested in characterizing clock gene expression and SBS in a model of acute colitis.

The nuclear receptor RORC1 has been described to be involved in circadian rhythm regulation, metabolic sensing and control of stress-induced-myelopoiesis in chronic inflammatory diseases. Thus, we hypothesize that inflammatory stress in the GIT might lead not only to clock gene disruption but also to an altered RORC expression, this might lead to a pathogenic myelopoiesis response, that might affect disease progression and resolution. Therefore, our second aim is to analyze emergency myelopoiesis response, in the context of acute colitis and study the role of RORC in disease pathogenesis, by the use of RORC deficient mice.

Chapter 3.

RESULTS

Chapter 3. RESULTS

3.1 A group of DSS treated mice develops severe disease course and wasting

To assess the development of acute colitis after 2.5 % DSS exposure, C57BL/6 mice were followed clinically and sacrificed at d7-d9 or d14. Colitis became evident from d5 of DSS treatment as assessed by body weight (BW) loss and clinical parameters (**Fig. 8A-C**). Mice with DSS-induced colitis developed signs of intestinal dysfunction and colitis, including hunched posture, ruffled fur, wet bloody or focal stained perianal area. These signs, as well as BW loss, peaked at d9 (**Fig. 8A-C**). However, while in about 80% of the animals the clinical symptoms spontaneously resolved, up to 20% of the mice developed severe BW loss (≥ 20%) (**Fig. 8B, C**),which subsequently led to death. Furthermore, these animals showed signs of body wasting such as shrinked spleen (**Fig. 10C**) and liver (data not shown) compared to other DSS treated animals at the same time point, which showed a BW loss <20%. Currently, 10% or greater involuntary BW loss (or < 20 BMI) is taken as a defining point for cachexia and it is correlated with decreased survival rate in patients with chronic inflammatory diseases (Wigmore et al., 1997). In non-cachectic animals, BW recovery to baseline levels occurred from d12-14. This event was in line with clinical improvement (**Fig. 8A-C**). Surprisingly, shortened colon length, a well-established parameter for gut inflammation, was not always significantly different when comparing non-cachetic to cachectic mice at d9. However, when taking into account all mice exposed to DSS, and untreated mice and performing a correlation of the colon length in function of the BW, linear regression revealed statistical significance ($p<o.ooo1$; $r^2= 0.7619$; **Fig. 9E**) Colon length of mice in clinical remission at d14 was significantly reduced compared to d7 (**Fig. 9A**). Histological scoring at d7 and d9 showed similar colitis severity in terms of leukocyte infiltration and epithelial damage, irrespective of the extent of BW loss (**Fig. 9B**). At d14 the histological score was still increased , but did not reach statistical significance.

IBD significantly reduces physical functioning. As expected, and as previously reported by others (Melgar et al., 2007), locomotor activity was dramatically decreased in DSS-treated mice at d8 in both cachectic and non-cachectic mice when compared to untreated H20 controls (**Fig. 8D**). Suppression of locomotor activity persisted in mice, which at d13 showed recovery from colitis associated diarrhea, bleeding and BW loss (**Fig. 8D**).

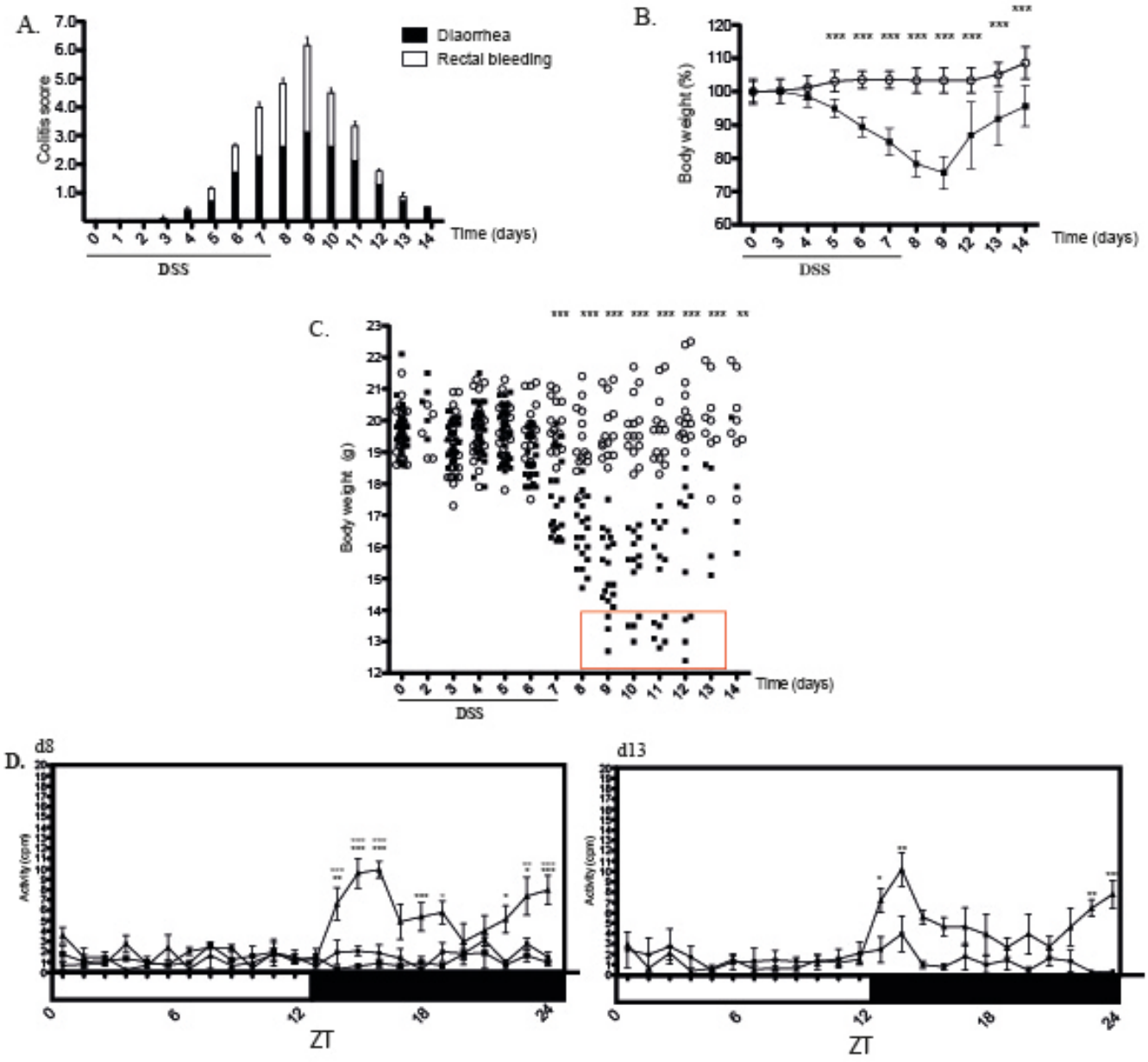


Figure 8. One subgroup of mice with DSS induced colitis develops cachexia.

(A) Colitis clinical score based on stool consistency (black bars) and presence of blood in stool (white bars) in mice exposed to 1 cycle of DSS treatment (day (d)0-7) and after DSS withdrawal receiving normal drinking water for additional 7 days (d8-14). Data are given as mean+/- SEM. n≥10 mice per time point.

(B). Relative loss of body weight in DSS colitis. The mean +/- SEM values of total body weight are given in percent relative to d0 of DSS treatment. Data are the result of one representative experiment out of 10 (n=15). H2O control (white circles); DSS (black circles). One-way ANOVA for repeated measurements. Bonferroni posthoc test: *** $p < 0.001$

C. Body weight loss in DSS colitis. Body weight in individual DSS animals (black circles) and untreated H20 controls (white circles) from a representative experiment out of 10, in n≥10 mice per time point is shown. DSS cachectic animals are delimited by a red square. Two-way ANOVA for repeated measurements. Bonferroni posthoc test ***: $p < 0.001$

D. Locomotor activity in DSS-induced colitis. Locomotor activity was assessed by implanting intraperitoneal (i.p) telemeters (TA-F10, DSI) in mice under isofluorane anesthesia, two weeks prior to acute colitis induction. Telemetry recordings were based on mean average counts per minute (cpm) of 1 h intervals by using the Dataquest A.R.T. software. Activity measurements were performed continuously during the whole course of the experiment. Two-way ANOVA for repeated measurements. Bonferroni posthoc test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2 Glycaemia in DSS-treated mice is reduced during acute colitis but returns to control levels in disease remission.

Assuming that animals, which developed a more severe disease course and body wasting, might suffer from an impairment in energy metabolism, we decided to evaluate their glucose levels in circulation. Prior to the study, initial blood glucose and BW were similar among the groups (data not shown). Melgar et al. reported in DSS treated mice decreased glucose circulating levels and fat mass, which the authors explained to be due to a decreased food intake and metabolic rate in DSS animals. As previously reported (Dong et al., 2013; Melgar et al., 2007), blood glucose levels were lower in DSS-treated mice than in untreated animals during the acute phase of the disease: 6.148±1.32, 6.063±1.13, 5.76±0.58 mmol/L at d7, d9 and d9 cachectic mice, respectively, versus 8.4±1.5, 7.27±1.12 and 7.863±1.29 mmol/L in water controls at d7, d9 and d14 respectively). However, glucose in blood increased to control levels at disease remission (d14, 8.144±0.76 mmol/L) (**Fig. 9D**). To our surprise, glycaemia in cachectic mice (d9) was not significantly lower than in non-cachectic animals in the acute phase of colitis (d9). However, linear regression and Pearson correlation analysis revealed a significant correlation between d7 blood glucose and absolut BW ($p=0.0039$, r^2 0.3095) in DSS treated animals. As expected, cachectic animals at d9 showed as well reduced glycaemia, which was correlated with a decreased BW ($p=0.0108$, r^2 0.5774). There was no correlation between blood glucose levels and BW in non-cachectic animals, as they showed higher BW than cachectic mice but glycaemia was not significantly different (**Fig. 9F**).

Our observation is supported by the work from Dong et al., who reported reduced levels of glucose in plasma, accompanied by an increase of amino acid, as well as metabolic intermediates from the TCA cycle in mice with acute colitis induced by 2.5 % of DSS for 7d. These data suggests that DSS causes a disturbance in lipid and glucose metabolism in mice (Dong et al., 2013).

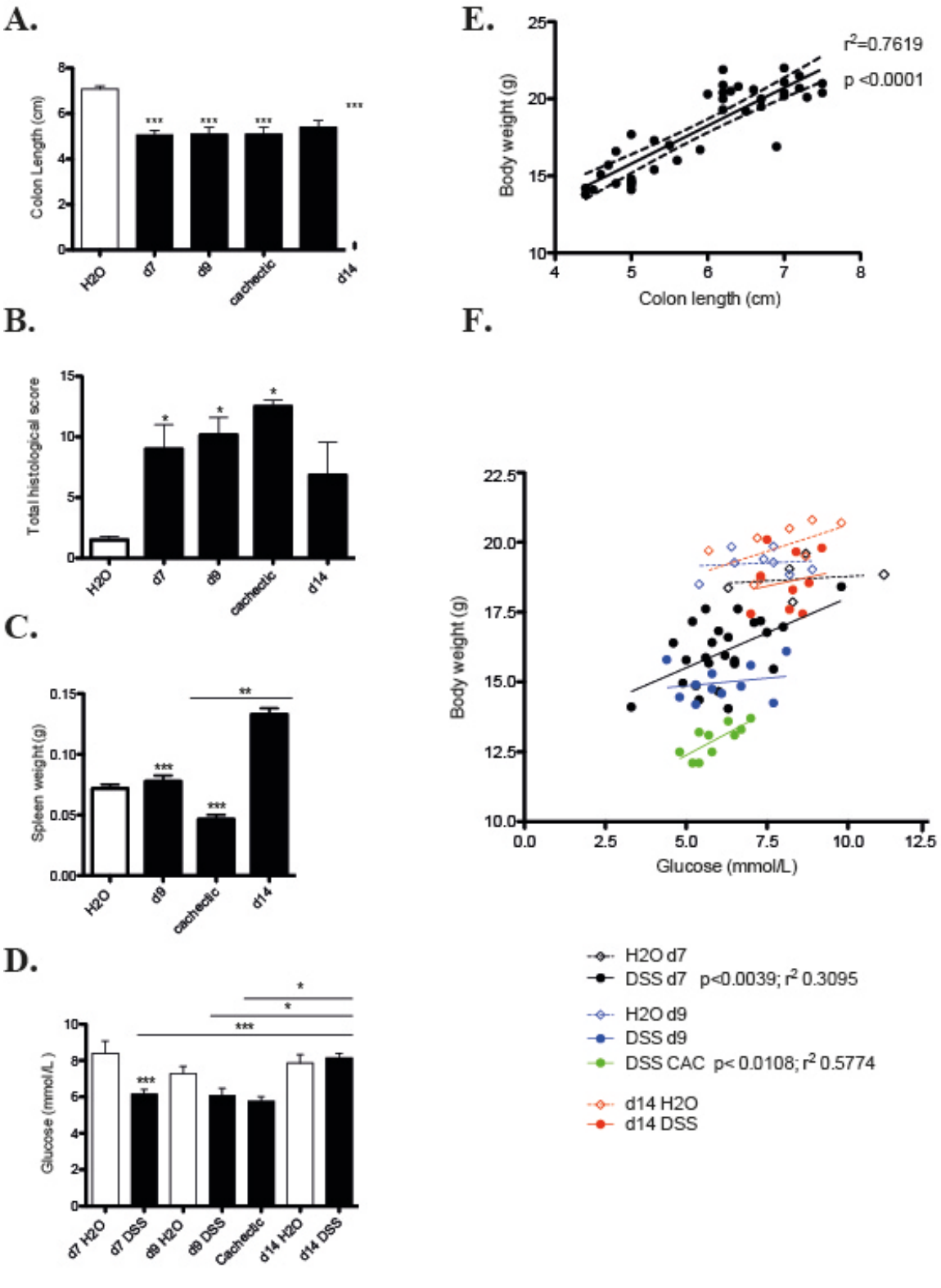

A.
Colon Length (cm)
H2O
d7
d9
cachectic
d14
B.
Total histological score
C.
Spleen weight (g)
D.
Glucose (mmol/L)
d7 H2O
d7 DSS
d9 H2O
d9 DSS
Cachectic
d14 H2O
d14 DSS
E.
Body weight (g)
Colon length (cm)
r²=0.7619
p <0.0001
F.
Body weight (g)
Glucose (mmol/L)
H2O d7
DSS d7 p<0.0039; r² 0.3095
H2O d9
DSS d9
DSS CAC p< 0.0108; r² 0.5774
d14 H2O
d14 DSS

Figure 9. Clinical parameters in DSS colitis

A. Colon length in DSS induced colitis.

Colon length was measured in DSS treated animals (black bars) at d7, d9 (cachectic and non-cachectic), and at d14, and in untreated control mice (white). Data shows the mean +/- SEM of triplicates from one representative experiment with $n \geq 5$ mice / group. One-way ANOVA. Bonferroni posthoc test; * $p < 0.05$; *** $p < 0.001$.

B. **Histological score in DSS colitis at d7, d9 and d14.** The histological score was assessed in both distal and proximal colon. DSS treated group (black); untreated (white); DSS cachectic mice (grey). $n \geq 3$ mice. One-way ANOVA. Bonferroni posthoc test. * $p < 0.05$.

C. Spleen weight in DSS induced colitis.

Spleen organs were harvested from DSS treated mice (black bars) at d7, d9 (cachectic and non-cachectic), and at d14, and in untreated control mice (white). Data shows the mean +/- SEM from one representative experiment with $n \geq 5$ mice/ group. One-way ANOVA. Bonferroni posthoc test; * $p < 0.05$; *** $p < 0.001$.

D. Blood glucose drops in DSS treated mice, but it levels restore in disease remission. Blood was collected from the distal tail without anesthesia with capillary test strips, and glucose concentration levels (mmol/L) were determined in DSS treated mice at d7, d9 (cachectic and non cachectic) and d14 (black bars), as well as in H2O controls (white bars) using a glucose monitoring system (Nova biomedical). Data shows the mean +/- SEM of $n \geq 5$ mice/ group. One-way ANOVA. Bonferroni posthoc test; * $p < 0.05$; *** $p < 0.001$.

E. Correlation between colon length and BW loss in DSS acute colitis.

The relation between colon length and absolut BW was calculated by Pearson correlation/ linear regression analysis between DSS treated mice at d7, d9 non-cachectic, d9 cachectic and d14, as well as untreated animals at d7, d9 and d15. Results were considered significant when $p<0.05$. R^2, coefficient of determination.

F. Correlation of body weight and blood glucose levels in DSS colitic mice.

The relation between blood glucose concentration and absolut body weight was calculated by Pearson correlation/linear regression analysis between DSS treated mice at d7, d9 non-cachectic, d9 cachectic and d14, as well as untreated animals at d7, d9 and d15. R^2, coefficient of determination. Results were considered significant when $p<0.05$. Untreated controls (open circles) ; DSS (black circles); DSS cachectic mice (grey circles).

3.3. Cytokine induced clock gene suppression (Ci-sCG) is reverted in colitis-associated cachexia

Having observed a difference in the disease course of mice with DSS induced colitis, we investigated whether this was reflected and possibly attributable to a difference in cytokine expression and in inflammation-associated dysregulation of clock gene expression. Thus we compared cachectic and non-cachectic mice at d9, and animals in clinical remission at d14.

In agreement with previous reports (Corazza et al., 1999; Ko and Chik, 2009; Ko et al., 2005; McAlindon et al., 1998; Neurath and Pettersson, 1997; Papadakis and Targan, 2000), colon mRNA expression levels of TNF-α and IL1-β were increased at d9 in DSS-induced colitis (**Fig. 10A**). However, differences in expression levels between cachectic and non-cachectic mice did not reach statistic significance. Surprisingly, expression levels of the aforementioned proinflammatory cytokines remained significantly up-regulated in the recovery phase of disease (d14) compared to water controls (**Fig. 10B**), although expression levels were overall lower than at d9. In contrast, TNF-α serum levels in DSS-cachectic subjects were significantly higher than in non-cachectic, although nor IL-1β was not detectable in any of the study groups (**Fig. 10C**).

Concomitant to increased TNF-α and IL1-β mRNA levels, IL-10 expression, was increased in non-cachectic mice, and even more pronounced in cachectic animals (**Fig. 10A**). Increased IL-10 mRNA expression persisted in the resolution phase of disease (**Fig. 10B**). Despite IL-10 was not detectable in any group in serum, we observed remarkably high levels of another member of the IL-10 family, IL-22 (**Fig. 10C**).

On the other hand and in contrast to the observations in colon tissue, TNF-α levels in serum were significantly reduced compared to cachectic mice, but no differences were observed with animals that did not develop DSS cachexia (**Fig. 10C**).

In line with this observation, we also found elevated serologic levels of IL-6 in the serum of mice at the peak of DSS-induced colitis (cachectic and non-cachectic; n.s. differences), although such high levels remained in colitis remitting animals (**Fig. 10C**). Importantly, IL-6 has been suggested as a key driver of cachexia, affecting mitochondrial respiration, with the subsequent effect in lipid mobilization and increased energy expenditure in WAT (Petruzzelli et al., 2014).

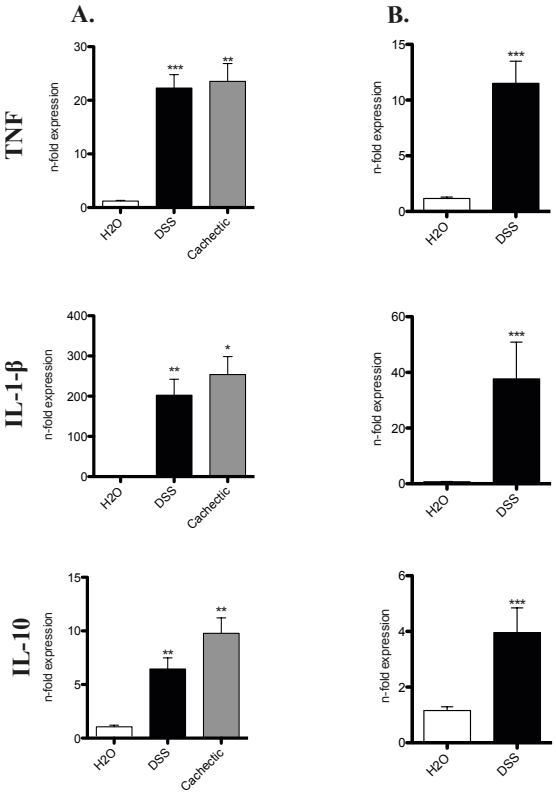
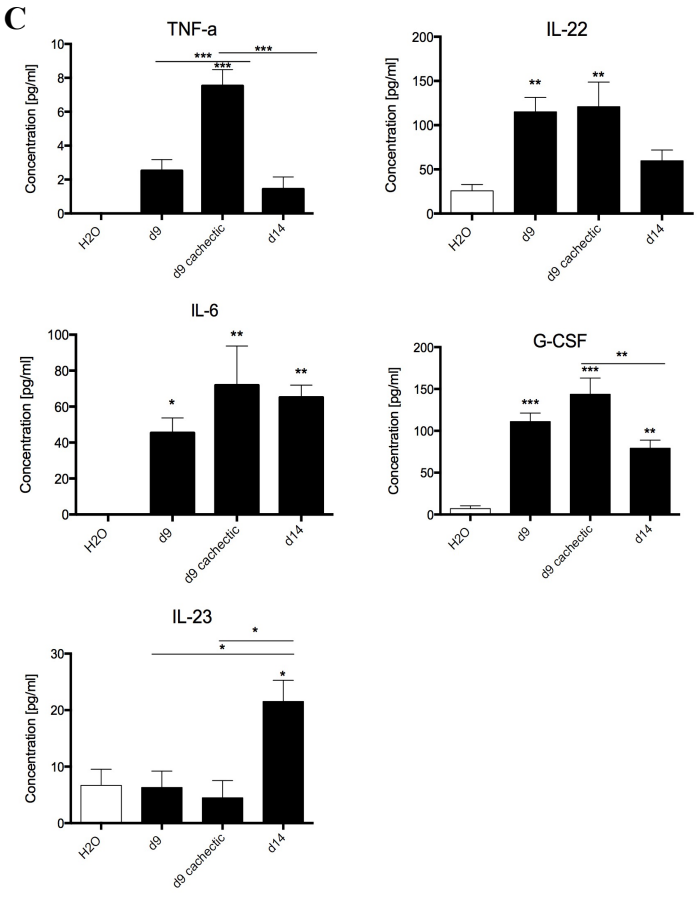


Figure 10. Cytokine gene expression in colon and blood of mice with DSS experimental colitis.

Expression of TNF-α, IL-1-β and IL-10 in DSS treated mice without cachexia (black bars) and with cachexia (grey bars) at d9 (A) and d14 (B). Data of RT-qPCR assays show the mean +/- SEM (error bars) comparing triplicates of DSS treated mice with H2O control. (C) Serum cytokine concentrations were determined using a Procarta 17-plex Immunoassay kit (Affymetrix, ebioscience) according to the manufacturer's protocol. The results represent one experiment with n ≥ 5 biological replicates / group. The mean is represented by +/- SEM. Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$



On the other hand, TNF-α and IL1-β have been reported to interfere with BMAL1-CLOCK activation of E-box dependent clock genes, resulting in suppression of the PAR-bZip clock-controlled genes (PAR-bZIP-CCGs) *Dbp*, *Tef*, and *Hlf* and the *Period* (*Per)1-3 genes in vitro* and *in vivo* (Cavadini et al., 2007). In accordance, we observed striking differences in clock gene colonic mRNA expression levels between DSS-cachectic and non-cachectic mice at d9 of DSS (**Fig. 11A**). Decreased expression of the core clock gene components, *Clock*, *Bmal1*, *Per3* as well as the PAR-bZIP-CCGs was observed in colon samples from DSS non-cachectic mice compared to untreated

animals (**Fig. 11A**). In contrast, the aforementioned clock expression pattern was lost in cachectic individuals, meaning that these mice showed similar expression levels than healthy controls. Less pronounced but still visible disruption of the molecular clock became also evident at the mRNA level in the spleen from DSS treated animals at d9, and as seen in the colon, no differences were observed in cachectic animals (**Fig. 12A**). However and to our surprise, animals in clinical remission at d14, showed persistent decrease of *Per3* and the PAR-bZIP-CCGs in colon (**Fig. 11 B**) and in spleen in a lesser extent (**Fig. 12B**). As mentioned above, this was associated with a significant increase in cytokine mRNA expression in the gut, compared to controls (**Fig. 11B**). Nevertheless, *Clock* and *Bmal1* colon expression levels at d14 did not differ from the levels observed in control mice (**Fig. 11**).

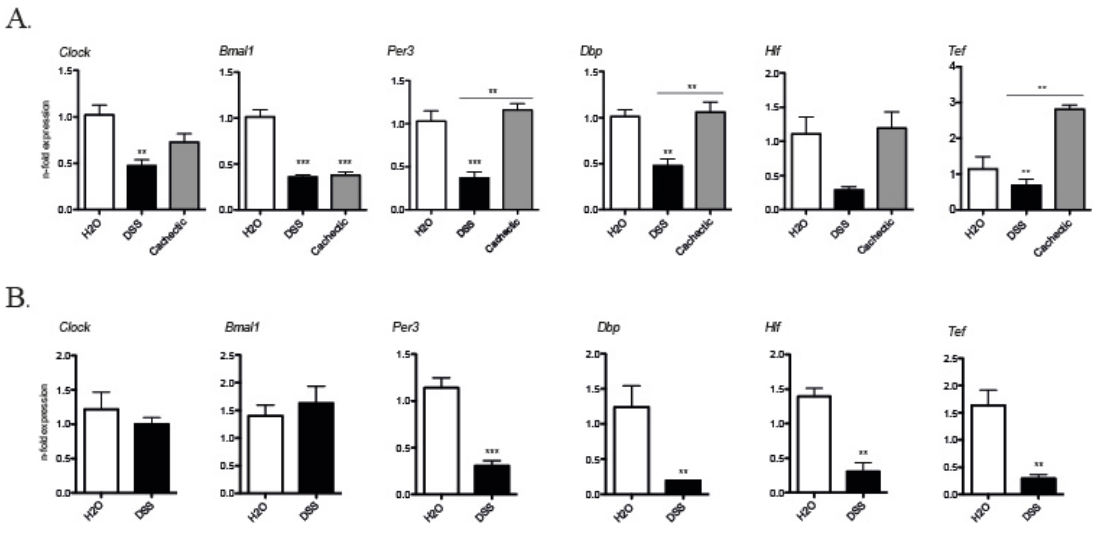


Figure 11. Clock gene expression in distal colon of DSS-induced-colitis.

Expression of *Clock, Bmal1, Per3, Dbp, Hlf* and *Tef* in DSS treated mice without cachexia (black bars) and with cachexia (grey bars) at d9 (A) and d14 (B). Data of RT-qPCR assays show one representative experiment out of 10 with n≥5 mice/ group. The results are given as the mean +/- SEM (error bars) comparing triplicates of DSS treated mice with H2O control (open bars). Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test; ** $p < 0.01$; *** $P < 0.001$.

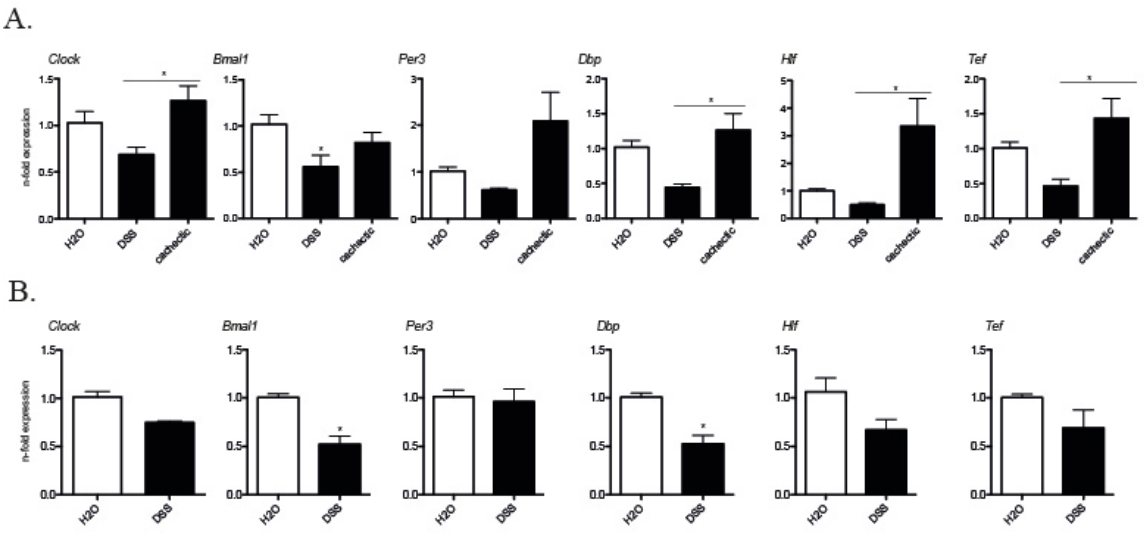


Figure 12. mRNA Clock gene expression in spleen from mice with DSS-induced-colitis.
Expression of *Bmal1, Clock, Per3, Cry1, Dbp, Hlf* and *Tef* in DSS treated mice (black) at d9 in non-cachectic and cachectic mice (A) and in mice showing a regain in body weight at d14 (B). RT-qPCR data are given as mean +/- SEM (error bars) of triplicates from one representative experiment out of 10 with n ≥ 5 mice/ group. One-way ANOVA. Bonferroni posthoc test, * $p < 0.05$.

Clock gene expression in sick animals might be constant during the circadian period, as we observed downregulation of clock genes in DSS treated animals at d7, both during the active (ZT12-18) and the resting period of the mice (ZT0-6). However, relative differences between experimental and control groups when assessing clock gene expression were higher at the onset of the resting period, regardless of the acrophase of the genes tested (data not shown). Importantly, the array of central and peripheral circadian clocks keeps a tight control of energy metabolism and nutrients intake, which does not occur indistinctly across the day, but periodically based on the species natural temporal niche. In general, the active period is associated with wakefulness, locomotion and increased food intake, thus high anabolic activity. Inversely, the resting period correlates with sleep and fasting, and mainly catabolic activity (Challet, 2013). As stated in the methods chapter, we performed all experiments starting at the onset of the resting period (7am), therefore a period with increased catabolism.

The difference in clock gene expression in colon samples harvested from non-cachectic and cachectic mice became also evident at the protein level. The amount of PER3 protein in distal colon was increased in cachexia compared to the detected amount in non-cachectic mice (**Fig. 13**).

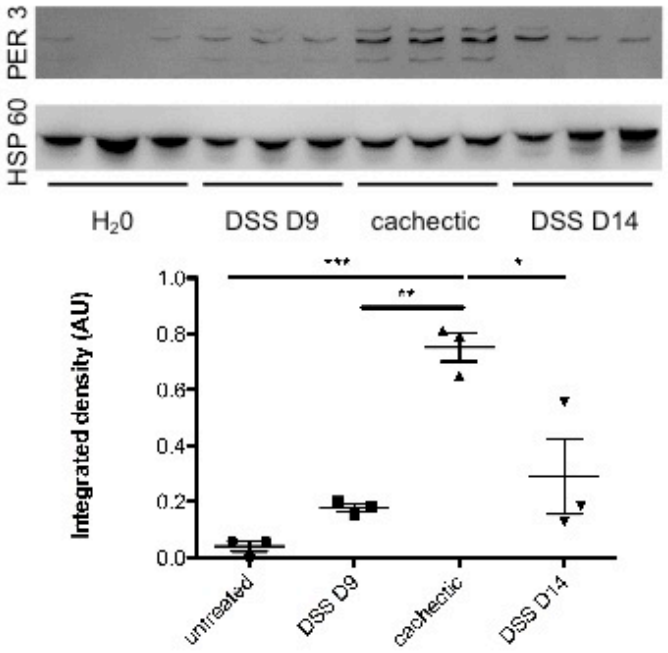


Figure 13. **Increased expression of PER3 in the spleen of cachectic mice.**

The presence of PER3 protein band at 132 kDa was detected in mouse distal colon. HSP 60 was used as a loading control. Quantification of integrated density revealed significant increase in PER3 protein concentration in the cachectic group (d9) when compared to the untreated group as well as to the DSS treated groups at d9 and d14. Values are given as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4. IL-10 deficiency does not prevent Ci-sCG in DSS cachectic mice

IL-10 is a predominantly anti-inflammatory cytokine with an essential role in maintaining GI homeostasis. Genetic variants in IL-10 or the IL-10 receptor (IL-10R) are associated with IBD susceptibility. IL-10-/- mice develop spontaneous colitis, and IL-10 deficiency exacerbates colitis in several models, including DSS and T-cell transfer colitis. Moreover, pharmacologically administered IL-10 ameliorates colitis in mice by inhibiting intestinal inflammation and suppressing proinflammatory cytokine production (Li et al., 2014). To date, a direct role of IL-10 in clock gene regulation has not been demonstrated, However, it is well-known that signaling through the IL-10R antagonizes proinflamatory signaling through various mechanisms, such as SOCS3 induction. Therefore, elevated IL-10 expression levels in colon of cachectic mice might mediate Ci-sCG reversion .To investigate a correlation between Il-10 expression and reversion of Ci-sCG, DSS colitis was induced in IL-10-/ and WT mice and disease course, locomotor activity and cytokine/clock gene expression was determined (data not shown). IL-10-/- animals showed accelerated disease onset (starting from d3-4 after DSS insult compared to d4-5 in their WT counterparts). Accordingly, KO mice showed more severe disease course in terms of diarrhea and rectal bleeding, as well as more pronounced BW loss %, as previously reported (Li et al., 2014). Further, reduction in locomotor activity at the dark phase of the 24-h cycle was evident at an earlier time point than in WT animals. Unfortunately, the majority of the animals had to be euthanized before the termination of the experiment (around d9) as they developed severe DSS cachexia. Nevertheless, IL-10 deficiency did not prevent reversion of Ci-sCG in IL-10-/- mice with cachexia (data not shown).

3.5 CisCG can not be reverted by systemic administration of TNF-α.

As mentioned above, TNF-α and IL1-β were reported to induce clock gene suppression i*n vitro* and *in vivo* in other models of inflammation (Cavadini et al., 2007). To investigate whether Ci-sCG expression patterns might relate to oscillations in systemic TNF-α expression levels. WT mice were treated with low and high dose of TNF- α. Injections were made at 2 different time points of the day : at ZT0 (onset resting phase, light) and at ZT12 (onset active phase; dark) (**Fig. 14**). It is known that mice exhibit a clock-controlled enhanced immune sensitivity to during their active phase (ahead of activity and feeding), since the expression of components of the innate immune system is then increased to anticipate for a higher risk of antigen encounter (Druzd and Scheiermann, 2013). The effect of time of day of TNF-α administration on lethal toxicity was examined previously by others (Hrushesky et al., 1994). Each mouse received a single i.p. Injection of 2ng or 60ng of m-TNF- α or PBS.

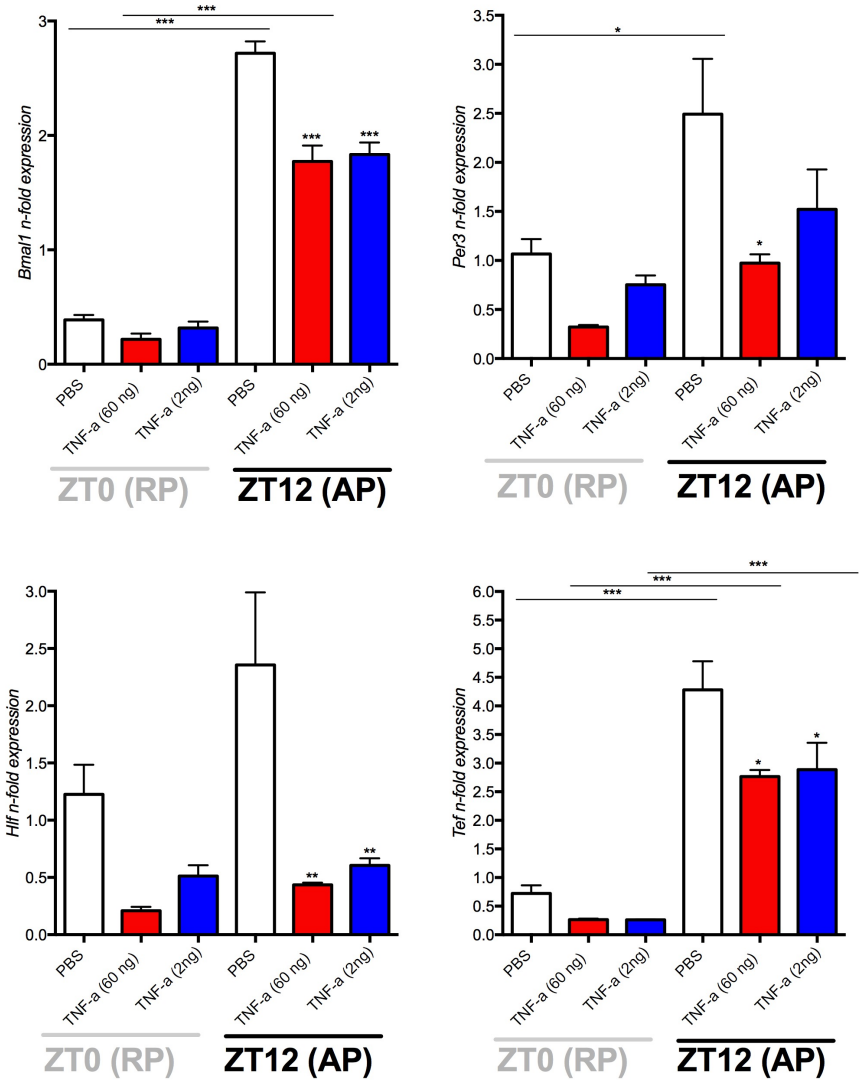


Figure 14. Ci-sCG develops in mice irrespective of the ZT chosed to inject TNF- α.

Recombinant murine TNF- α was injected intraperitoneally (i.p.) in female WT naïve mice at ZT0 (onset resting period) or ZT12 (onset of activity period). Two doses of TNF- α were used: 2ng (blue) or 60 ng (red). PBS was injected as a control. Expression of clock genes was assessed in spleen harvested after 12h post TNF- α injection. RT-qPCR data are given as mean +/- SEM (error bars) of triplicates from one with n ≥ 5 mice / group. One-way ANOVA. Bonferroni posthoc test, * $p < 0.05$, *** $p < 0.001$

BW loss was monitored hourly for 12h and they were sacrificed. As expected, regardless of the TNF-α dose administered, animals who received TNF-α injection 1h ahead of the onset of the active phase (ZT11) exhibited accelerated BW loss and small intestine edema (data not shown). On the other hand, animals that were injected 1h ahead of the resting phase onset (ZT23) showed signs of intestinal edema later time post-injection, decreased weight more gradually. Nevertheless, higher TNF-α dose was not reflected in the expected differences in clock gene level in the spleen. We did not observe reversion of Ci-sCG in mice injected with the higher dose, as hypothesized. Instead, we observed suppression of clock gene expression independently of the TNF-α i.p. dose, although the extent of suppression was more remarkable at higher TNF-α dosage (**Fig. 14**). Thus, after TNF-α injection BW loss correlated with Ci-sCG, regardless of the dose.

3.6. Absence of cytokine induced suppression of clock genes (Ci-sCG) in splenic Ly6C^{hi} from cachectic mice.

IBD is recently viewed as a dysregulated innate immune response with activation of transcriptional networks involved in leukocyte priming, activation and recruitment (Rahman et al., 2010). It is known that the differentiation program of patrolling Ly6C^{hi} monocytes is able to adapt in situations of increased demand, in acute inflammation, as well as to initiate resolution.

Nguyen et al. reported that Ly6C^{hi} inflammatory monocytes exhibit a cyclic pattern of trafficking that confers protection against *Listeria monocytogenes* in mice, and which is and is regulated by the repressive activity of the circadian gene Bmal1. Accordingly, myeloid cell-specific deletion of Bmal1 induces expression of monocyte-attracting chemokines and disrupts rhythmic cycling of Ly6Chi monocytes, predisposing mice to development of pathologies associated with acute and chronic inflammation (Nguyen et al., 2013).

We hypothesize that the molecular clock might have a role in controlling immune responses in order to reestablish immune homeostasis in inflammatory diseases. In order to investigate this hypothesis, we isolated inflammatory monocytes from the spleen of animals with acute colitis at different and analyzed clock gene expression. Splenic CD45^{+}CD11b^{+}Ly6C^{hi} monocytes were isolated at the peak of disease at d9 (from cachectic and non-cachectic animals) and in disease remission at d14 (**Fig. 15A**).

In accordance with our findings in total tissue (colon and spleen) expression of the core clock gene *Clock*, *Per3* and PAR-bZIP-CCGs was suppressed in splenic Ly6C^{hi} monocytes obtained from mice with DSS acute colitis at d9 in comparison to monocytes obtained from untreated animals. Colitis-associated suppression of the molecular clock persisted in inflammatory monocytes obtained from mice in remission

at d14 (**Fig. 15B**). Importantly, Ci-sCG was lost in Ly6C^{hi} monocytes harvested from the spleen of cachectic mice, approaching the expression levels observed in splenic monocytes from water controls (**Fig. 15B**).

In summary, the mRNA Ci-sCG signature observed at the inflammation site, in the colon of DSS-treated animals, was also visible in their myeloid compartment, specifically in the Ly6C^{hi} inflammatory monocyte subset present in the spleen. Taken collectively, these data suggests that Ly6C^{hi} inflammatory monocytes exhibit characteristic circadian clock gene expression patterns during the course of acute colitis.

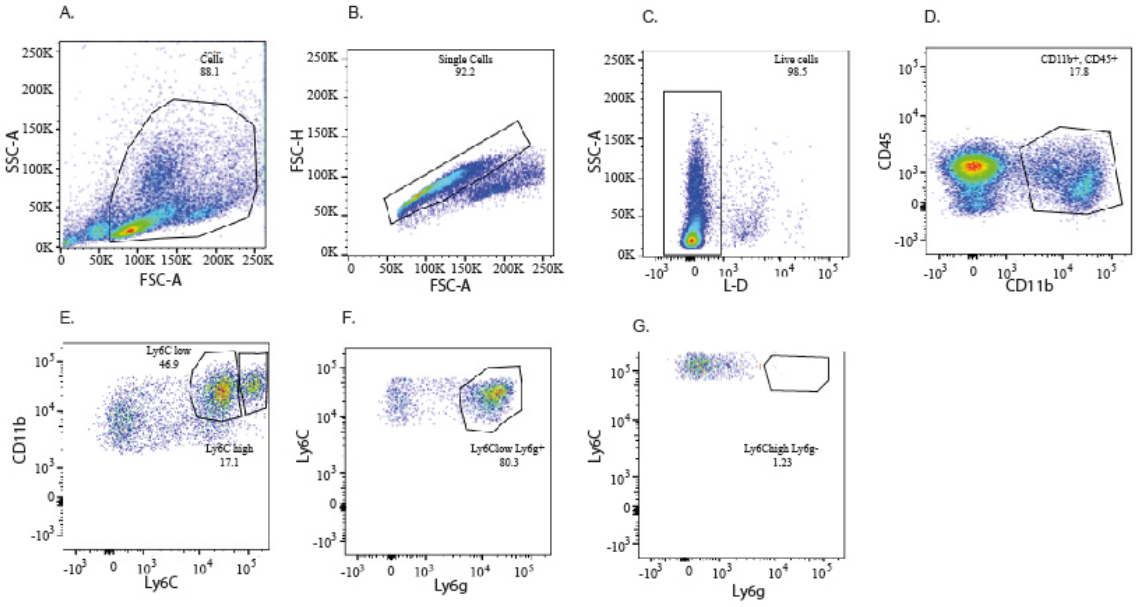
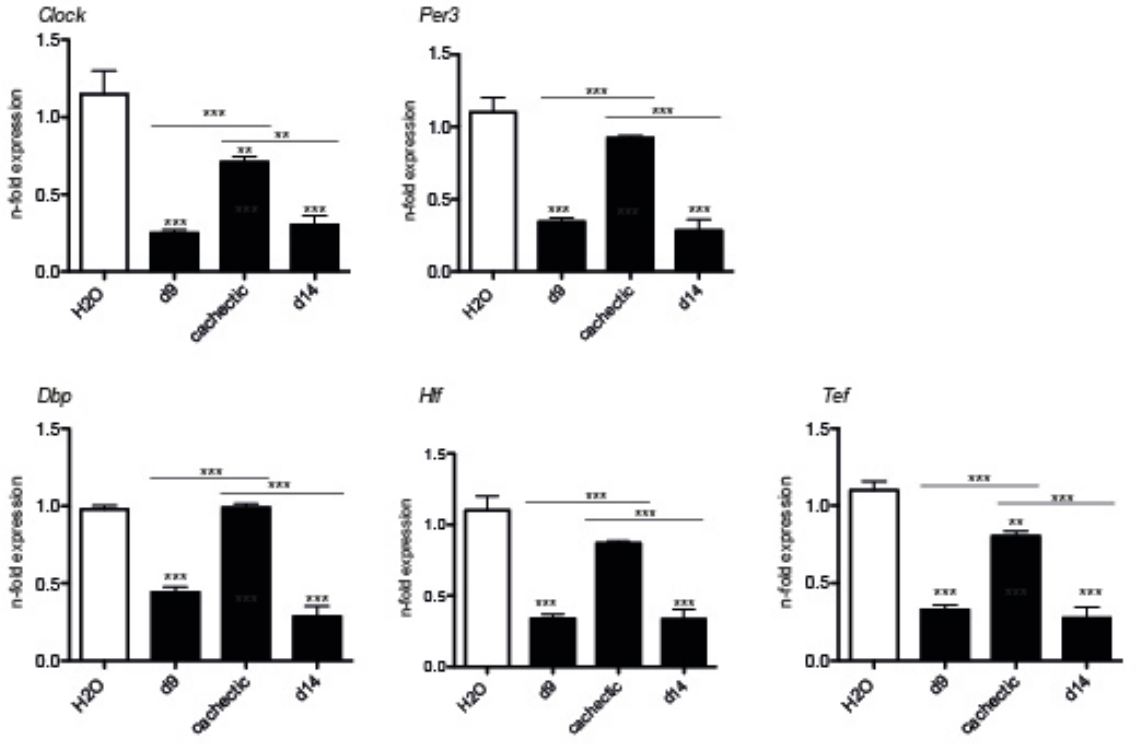


Figure 15. Ci-sCG reversion is observed in inflammatory monocytes from the spleen of mice with DSS-associated cachexia.

A. Sorting strategy used for isolation of splenic inflammatory monocytes (CD11b^{+}Ly6C^{hi}) from mice with DSS-induced-colitis. Splenocytes from DSS treated or H2O control mice were isolated at d9 or d14 and sorted using a FACS Aria III instrument. FACS Dotplots presented here show splenocytes from DSS treated mice at d9. Debris (SSC-A vs. FSC-A, A) and doublets (FSC-H vs. FSC-A, B) were excluded and live/dead discrimination was determined using the amine reactive far red (SSC-A vs. L_D, C). CD45^{+} CD11b^{+} myeloid cells (D) were then sub-gated on CD11b^{+}Ly6C^{+} cells (E), followed by excluding Ly6G^{+}cells (F). Inflammatory monocytes identified as CD11b^{+}Ly6C^{hi}Ly6G^{-} (E, G), were FACs-sorted for gene analysis.



B. Clock gene expression in splenic CD11b^{+}Ly6C^{hi} inflammatory monocytes in DSS-induced-colitis.
Expression of *Clock, Per3, Dbp, Hlf* and *Tef* in monocytes obtained from control H2O treated mice (open bars) and DSS treated mice (black bars): cachectic and non-cachectic mice at d9, and mice showing a regain in body weight at d14. RT-qPCR data are given as mean +/- SEM (error bars) of triplicates from one representative experiment with n≥5 mice/ group. One-way ANOVA. Bonferroni posthoc test: ** $p < 0.01$; *** $p < 0.001$.

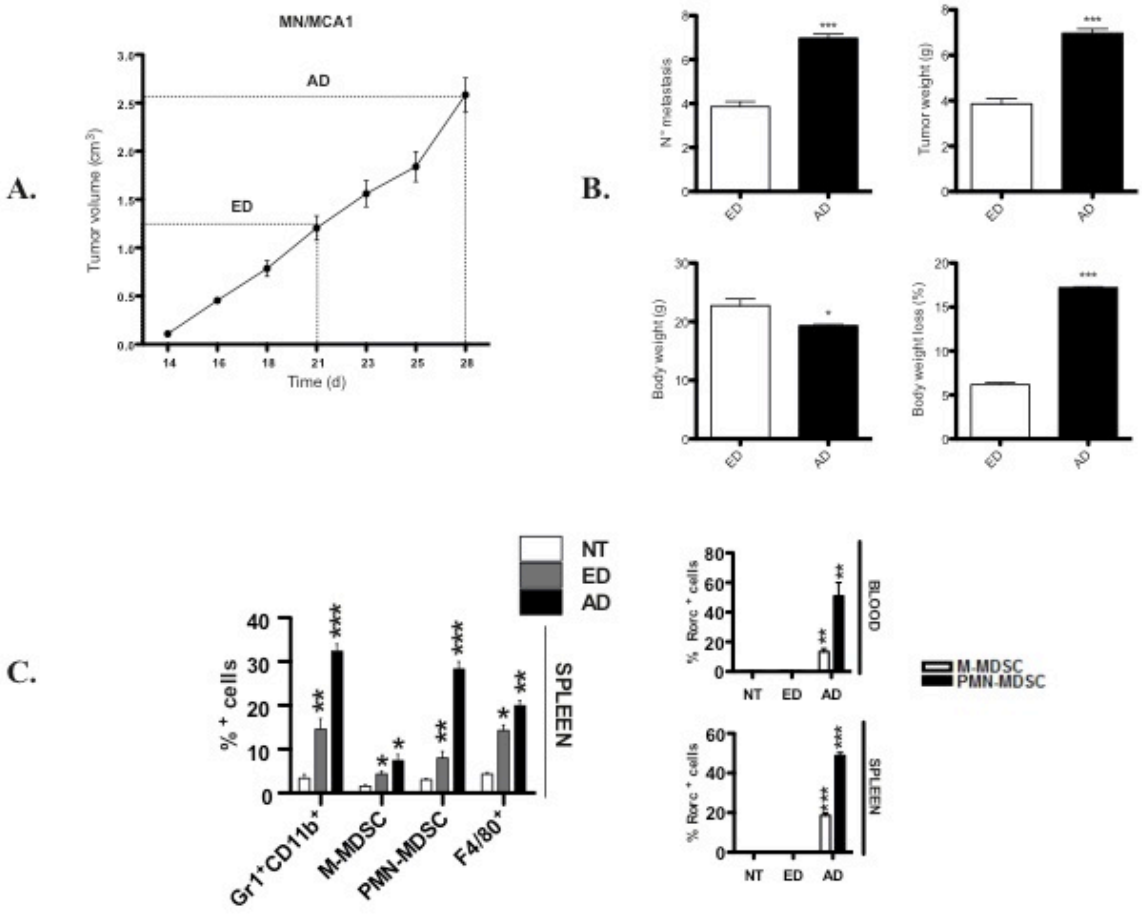
3.7. Tumor associated macrophages from advance cancer stage show reversion of Ci-sCG.

To investigate whether reversion of Ci-sCG might constitute a characteristic feature of chronic inflammation-associated cachexia, we investigated clock gene expression in a model of cancer cachexia. We screened the transcriptional level of expression of several clock genes in fibrosarcoma (MN/MCA1)-bearing C57BL/6 mice. A tumor volume of <1.5 cm^3 and <5 lung metastases, at days 21-23 post-tumor-injection, was defined as early-stage disease (ED), whereas tumors larger than 2 cm^3 and a > 5 metastasis at days 24-28, was defined as advance-stage disease (AD) (Fig. **16A-B**).
BW loss in AD was 17.2 ± 0.2 % compared to 6.2 ± 0.4 % in ED tumor bearing mice (**Fig. 16B**).
As previously described in AD fibrosarcoma tumor bearing mice (Strauss et al., 2015a), FACS analysis confirmed that the major splenic myeloid population from the CD45^{+}CD3^{-} cell pool was CD11b^{+}GR1^{+}, comprising a predominant PMN population characterized by the expression of CD11b^{+}GR1^{+}Ly6Ghi Ly6Clo (PMN-MDSCs) and a monocytic population defined as CD11b^{+}GR1^{+} Ly6Chi Ly6Glo (M-MDSCs), as well as F4/80^{+} macrophage populations (**Fig. 16C-D**).

As a first attempt we analyzed clock gene and cytokine gene expression by RT-qPCR in different tissues (MN/MCA1 fibrosarcoma cells, liver, spleen and TAMs) obtained from tumor bearing mice with ED or AD. When analyzing the expression of *Per 1-3* and PAR-bZIP-CCGs genes, we observed higher level of mRNA expression in AD compared to ED in fibrosarcoma cells MN/MCA1, liver, spleen (data not shown) and TAMs (**Fig. 17**), confirming our results in DSS cachexia. Interestingly, the level of expression at ED vs AD was significantly higher in TAMs compared to the other organs (data not shown). This suggests a possible dilution of the difference in gene expression when analyzing total tissue versus a specific myeloid population (**Fig. 17**).

In addition, we tested the core clock genes *Bmal1, Clock and Npas2*. Interestingly, no differences were observed between ED and AD in the core components Clock and Bmal1, but only in neuronal PAS domain protein 2 (Npas2, paralog of Clock in several tissues) in TAMs (**Fig. 17**) as well as in the other tissues tested (data not shown).

Furthermore, we observed higher pro-inflammatory mRNA expression (*TNF-α, IL-6, IL-1β*) as well as a remarkable increased in *IL-10* expression, in AD TAMs compared to ED TAMs (**Fig. 17**). Finally, we observed an up-regulation of *Rorc1* gene in TAMs from AD tumor bearing mice, as well as *Irf8* expression (**Fig. 17**). This observation correlates with a body weight loss > 15 % in mice with AD (**Fig. 16A**) suggesting that acute metabolic changes might impact on circadian control of myeloid immune responses.



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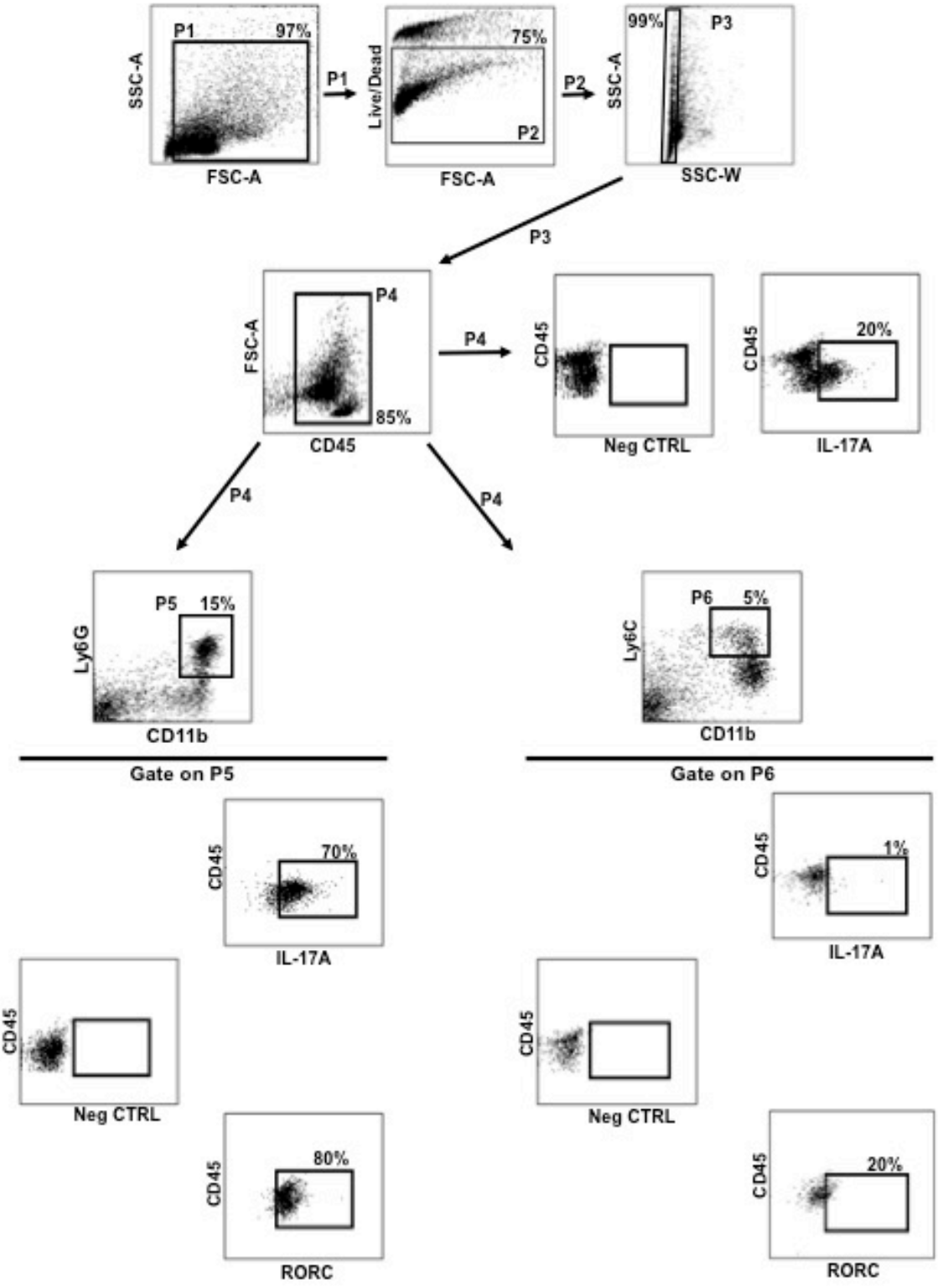
P1
97%
SSC-A
FSC-A
P1
75%
Live/Dead
P2
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P4
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85%
CD45
P4
CD45
Neg CTRL
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CD45
IL-17A
P4
P4
P5
15%
Ly6G
CD11b
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5%
Ly6C
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CD45
IL-17A
CD45
Neg CTRL
80%
CD45
RORC
1%
CD45
IL-17A
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Neg CTRL
20%
CD45
RORC

Figure 16. Advance cancer disease is associated with an expansion of RORC^{+} MDSCs in the spleen of WT tumor bearer mice.

A. Murine fibrosarcoma (MN/MCA1) cells (10^5) were inoculated i.m. in the left hind limb of female WT mice .Tumour growth was monitored three times a week with a calliper, starting from day 14. After tumour cell injection (day 0), tumour volume (cm^3) was measured starting from day 14 until day 28. MN/MCA1 bearing mice were classified into early-(ED) and late-stage (AD) tumour bearers.

B. Clinical parameters **(**BW (g), BW loss, tumour weight (g) and n° of lung metastasi) were evaluated in WT ED (white) and AD (black) tumour bearing mice.

C. Mouse tumours were cut into small pieces, disaggregated with collagenase (0.5mg/mL) and filtered through cell strainers. in order to obtain cell suspension, which was used for further procedures. Cell suspensions (10^6) were stained with specific antibodies and with live/dead dye (Invitrogen, Life Technologies) and analysed by FACS instrument. Splenic % of CD11b^{+}Gr1^{+}, M-MDSCs, PMN-MDSCs and F4/80^{+} macrophages was analysed in AD (black bars), ED (grey bars) and healthy controls (NT, white). % of RORC+ cells was assessed in PMN-MDSCs (black bars) and M-MDSCs (white bars) in blood and spleen from ED and AD tumour-bearing mice and in healthy controls

D. Gating strategy for splenic MDSCs and RORC/IL-17A expression .

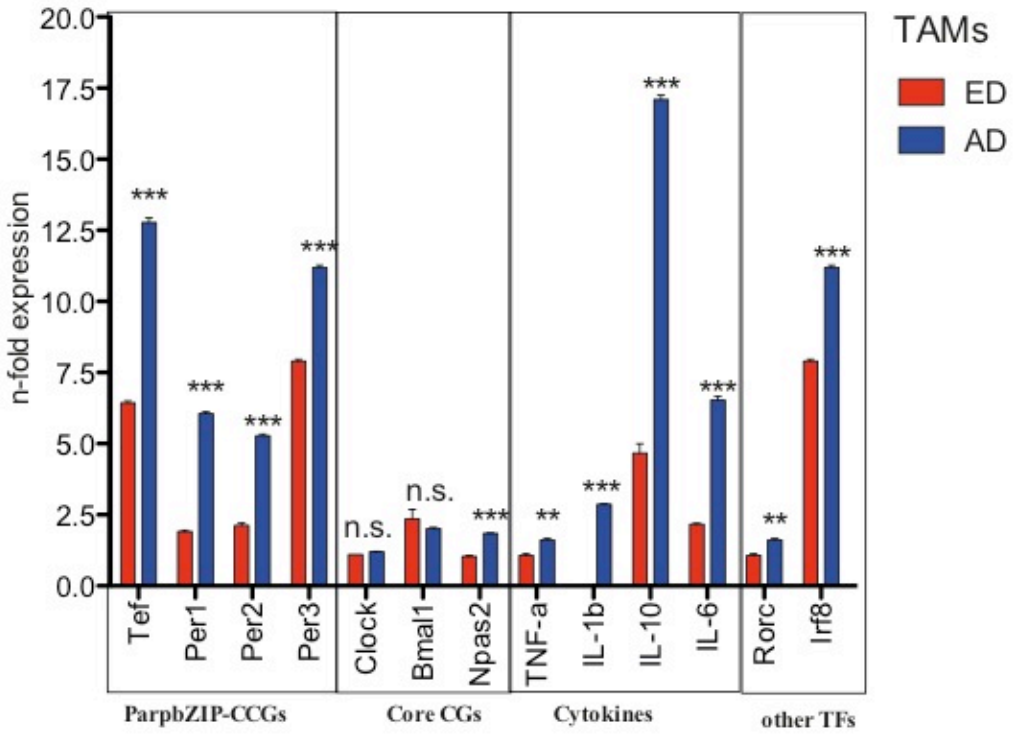


Figure 17. Gene expression in TAMs from ED or AD tumor-bearing mice.

Quantification of *Clock, Bmal1, Npas2, Per1-3, Dbp, Tef, TNF-a, IL-1b, IL-6, Rorc* and *Irf8* mRNA expression was assessed in TAMs from ED (red) or AD (blue) tumor-bearing mice. Data of RT-qPCR assays show one experiment with n≥5 mice/ group. The results are given as the mean +/- SEM (error bars) of n-fold mRNA expression, comparing ED TAMs versus AD TAMs. Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test; ** $p < 0.01$; *** $P < 0.001$

3.8 Ci-sCG reversion in DSS cachexia correlates with induction of the G-CSF/CXCL12 axis and systemic DNA damage response.

We have observed that Ci-sCG in myeloid cells correlates not only with acute colitis, but most importantly also with colitis remission. The circadian TFs DBP, HLF and TEF implicated in Ci-sCG in colitis remission are described to be involved in gene repair and drug metabolic detoxification (Gachon et al., 2011) This correlation suggests that Ci-sCG might be required for resolution of myeloid inflammation.

WT mice were given 2.5% DSS for 7 days followed by a 7-day recovery. Colitis was assessed by BW loss, clinical colitis score (stool consistency and rectal bleeding) and histopathology. We compared cachectic and non-cachectic mice reaching the peak of DSS-induced acute colitis at d9 and after 7 days of recovery, at d14, in the resolution phase of gut inflammation. RT-qPCR analysis of spleen and colon (data not shown) segments showed a dramatic increase in *G-csf* and *Cxcl12* in cachectic mice, whereas no significant expression in comparison to untreated H20 controls was observed in mice with acute colitis at d9 or in colitis remission at d14 (**Fig.18**). In addition, mice with acute colitis at d9 and cachectic mice show an important rise in *Il-17a* expression levels (**Fig.18**).

Whereas the differences noted in the expression of *Gm-csf* were not impressive, we detected a striking increase in *M-csf* expression in spleen of cachectic mice (**Fig. 18**) but it was not detected in the colon (data not shown). Increased M-CSF levels in response to DNA damage resulting in enhanced recruitment of MO- and PMN- MDSCs have been described (Mills et al., 2015), suggesting a crucial role for DNA damage responses in mediating recruitment and functional polarization of myeloid subsets.

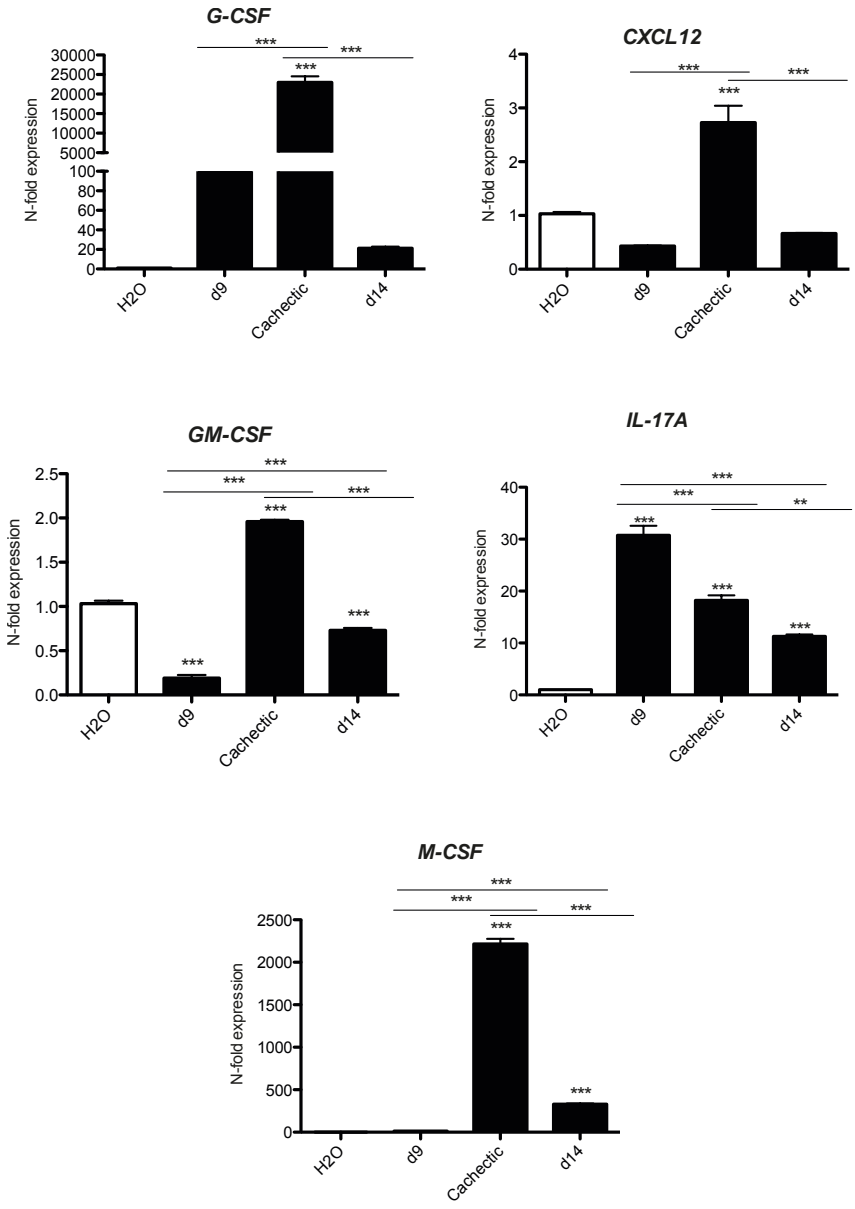


Figure 18. Myelopoiesis related growth factors and cytokine expression in spleen of DSS treated animals.

Relative mRNA expression of *g-csf, cxcl12, gm-sf, m-csf and il-17A* was measured by RT-qPCR in the spleen of DSS treated mice without cachexia and with cachexia at d9 and d14 (black bars) in comparison with healthy controls (white bars). Data of RT-qPCR assays show one representative experiment out of 3 with n≥5 mice/ group. The results are given as the mean +/- SEM (error bars) of n-fold expression, comparing DSS treated mice with H2O control. Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test; * $p < 0.05$, ** $p < 0.01$; *** $P < 0.001$

3.9. Cachexia extinguishes monocyte production and promotes neutrophil infiltrates

It remains unknown whether the circadian epigenetic control of the transcriptome in innate immune cells might affect the emergency myelopoiesis response in terms of phenotype and cell numbers. In order.to examine this process, whereby the host replenishes peripheral missing myeloid populations following the initial inflammatory insult, we compared the kinetics of $Ly6C^{hi}$ monocytes and $Ly6G^{+}Ly6C^{int}$ neutrophils in spleen and colon of mice with acute colitis at d7, d9, mice without colitis cachexia and mice in colitis remission at d14 .

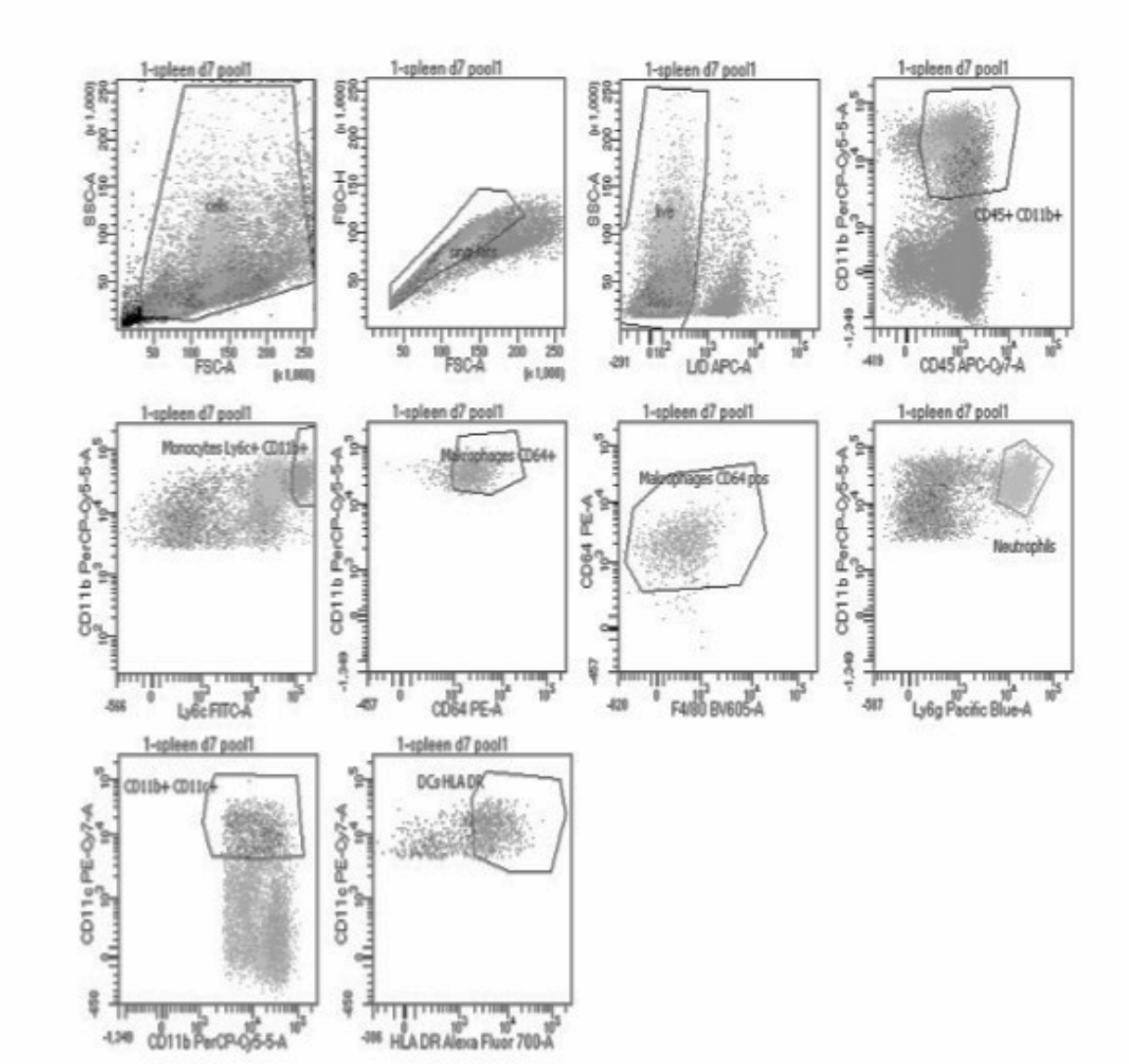


Figure 19. Sorting strategy myeloid subsets in DSS colitis

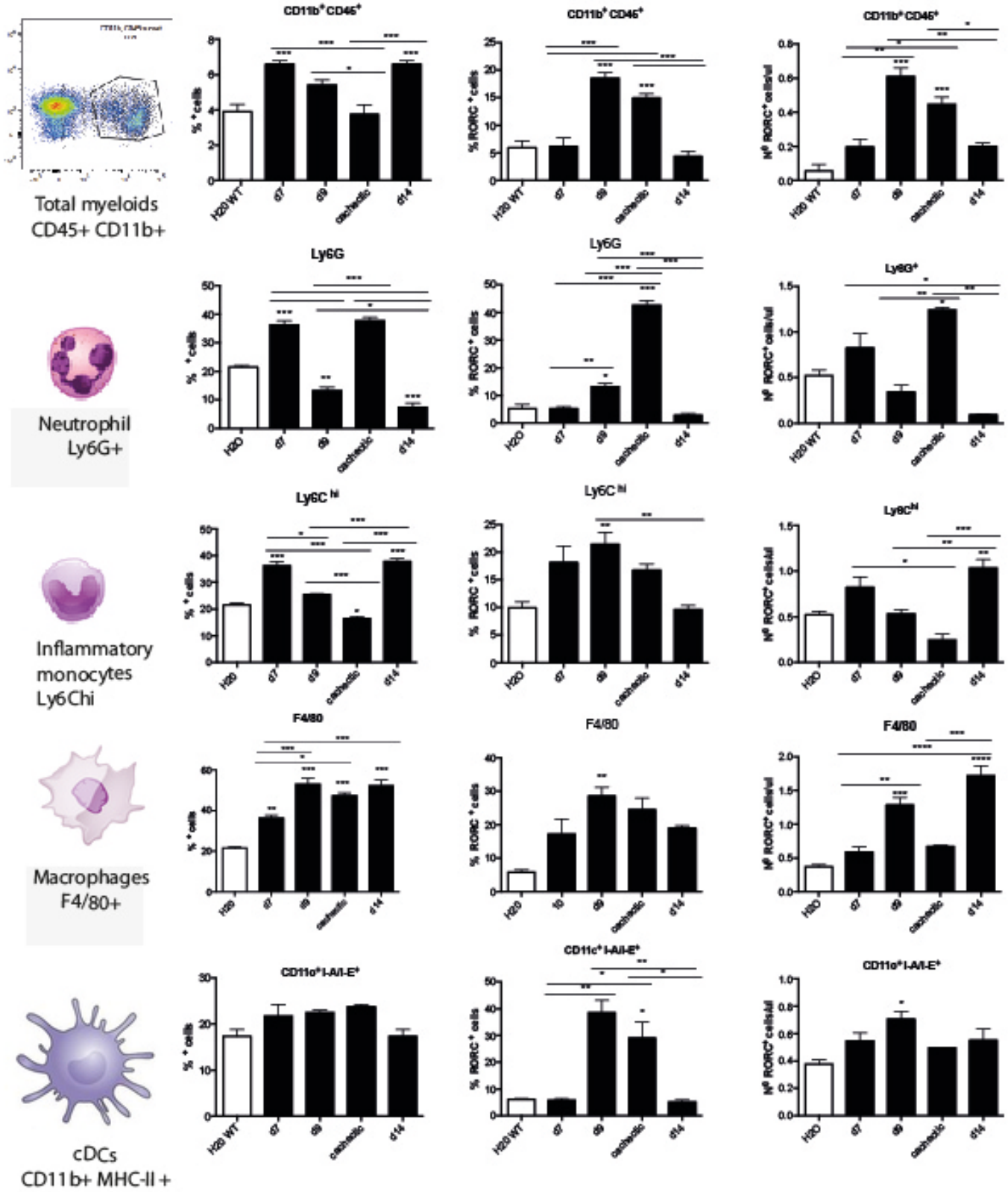
Splenocytes from DSS treated or H2O control mice were isolated at d9 or d14 were analyzed by FACS Aria III instrument. FACS Dot plots presented here show splenocytes from DSS treated mice from a representative experiment out of 3. Debris (SSC-A vs. FSC-A, A) and doublets (FSC-H vs. FSC-A, B) were excluded and live/dead discrimination was determined using the amine reactive far red (SSC-A vs. L/D, C). $CD45^+$ $CD11b^+$ myeloid cells were then sub-gated on $CD11b^+Ly6C^{hi}$ cells, $Ly6G^+$ $Ly6C^{int}$cells, $CD11c^{hi}$ MHC-class II^+ DCs and $F4/80^+$ $CD64^+$ macrophages.

$Ly6G^{hi}$ neutrophils were significantly increased in all study groups when compared to healthy controls in colon, whereas in spleen there was an increase at d7, but the levels decrease at d9 an remained low in remission (**Fig. 20, 21**). Importantly, BW loss in colitis cachexia correlates with a massive increase in $Ly6G^{hi}$ neutrophils in spleen (**Fig. 20**).

In contrast, as shown in **Fig. 20 and 21**, DSS-induced-colitis cachexia is associated with a decrease in $Ly6C^{hi}$ monocytes in spleen in comparison to healthy H20 controls, whereas a significant increase in $Ly6C^{hi}$ monocytes in spleen correlates with evident BW regain in mice in colitis remission (d14). Of note, we observed increased serum levels of IL-23 at d14, which has been reported to increase monocyte recruitment (Indramohan et al., 2012) **(Fig. 10D).** While mice at d7 of acute colitis exhibit a rise in splenic inflammatory monocytes in comparison to healthy controls, non-cachectic mice with acute colitis at d9 (2 days post DSS withdrawal) already fail to exhibit an increase in inflammatory monocytes (**Fig 20**). However, inflammatory monocytes peak at d9 of acute colitis in the colon from non-cachectic mice (**Fig. 21**).

On the other hand, we did not observed differences in APC populations like macrophages or in DCs. Although we observed an increase of the $F4/80^+$ macrophage % from d7 in all DSS treated groups compared to the H2O controls, no differences were seen between d9 cachectic and non-cachectic mice, nor in colitis resolution. Finally, we analyzed $CD11c^{hi}$ MHC Class II^+ DCs without any differences in the percentage of the population between DSS and untreated mice (**Fig. 21**).

Figure 20. Myeloid population dynamics in the spleen of DSS-induced-colitis mice Splenic myeloid cells were sorted, as described in experimental procedures and in figure 23 (gating strategy). The results are given as the mean +/- SEM (error bars). DSS treated animals (black bars) and untreated mice (white bars). Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test; ** $p < 0.01$; *** $P < 0.001$


% + cells
% RORC + cells
N° RORC+ cells/uL
SPLEEN
Total myeloids
CD45+ CD11b+
Neutrophil
Ly6G+
Inflammatory
monocytes
Ly6Chi
Macrophages
F4/80+
cDCs
CD11b+ MHC-II +
CD11b+ CD45+
Ly6G
Ly6C hi
F4/80
CD11c+ I-A/I-E+
H20 WT
d7
d9
cachectic
d14

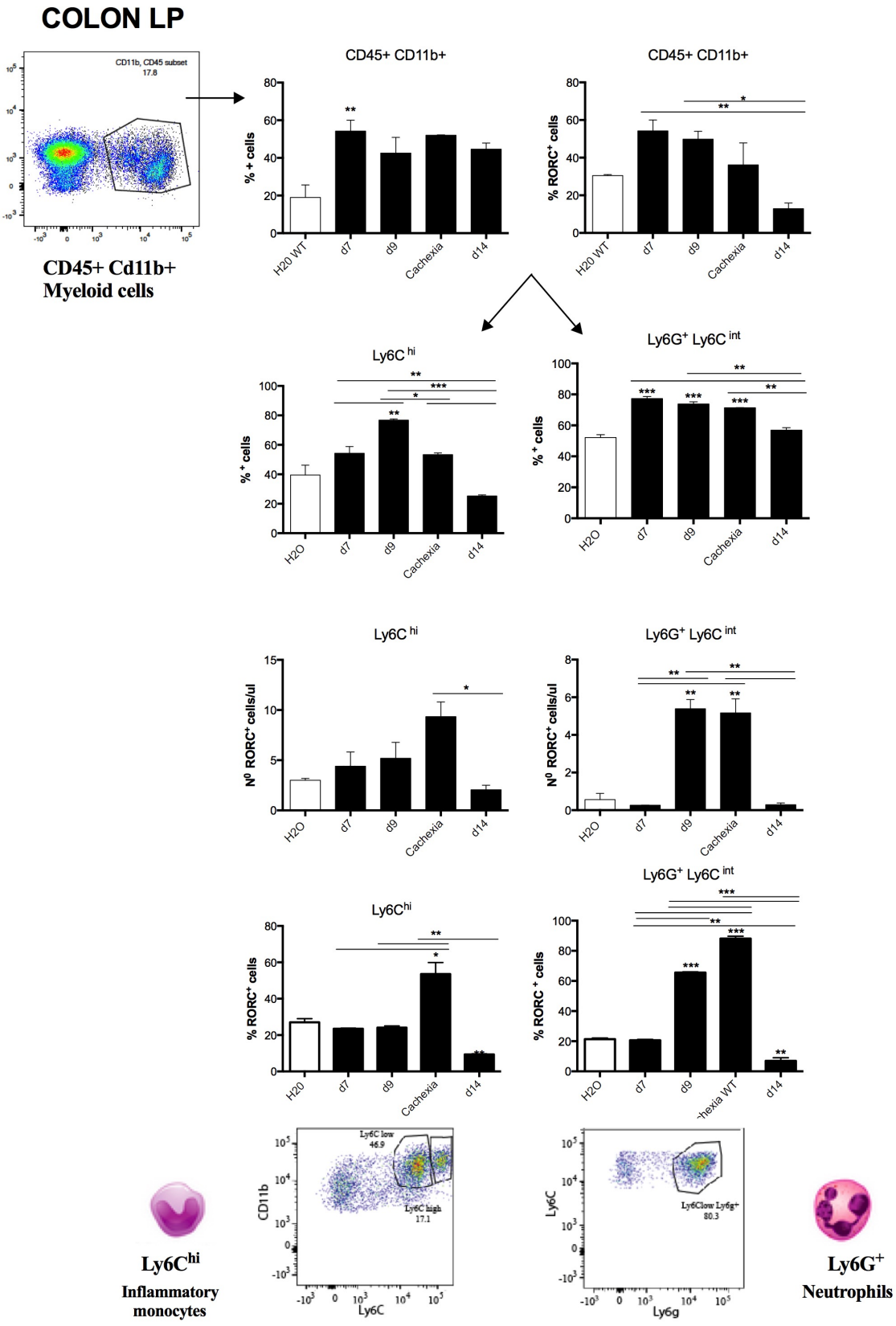

COLON LP
CD11b, CD45 subset
17.8
CD45+ Cd11b+
Myeloid cells
CD45+ CD11b+
% + cells
H20 WT
d7
d9
Cachexia
d14
CD45+ CD11b+
% RORC+ cells
Ly6C hi
% + cells
H2O
Ly6G+ Ly6C int
% + cells
Ly6C hi
N0 RORC+ cells/ul
Ly6G+ Ly6C int
N0 RORC+ cells/ul
Ly6Chi
% RORC+ cells
Ly6G+ Ly6C int
% RORC + cells
Ly6C low
46.9
Ly6C high
17.1
CD11b
Ly6C
Ly6Clow Ly6g+
80.3
Ly6C
Ly6g
Ly6Chi
Inflammatory
monocytes
Ly6G+
Neutrophils

Figure 21. Myeloid population dynamics in the colon of DSS-induced-colitic mice.

Myeloid cells from colon LP were isolated, as described in experimental procedures and in **Fig. 19** (gating strategy). Colonic % of $CD11b^{+}CD45^{+}$, $Ly6C^{hi}$ $Ly6G^{-}$ and $Ly6G^{+}$ $Ly6C^{int}$ was analysed in DSS treated animals, including d9 cachectic mice (black bars), and healthy controls (white bars). Percentage (%) and absolute number (n°$RORC^{+}$ cells/µL) of $RORC^{+}$ cells was assessed in both DSS (black) and untreated mice (white) .Dot plots show one representative experiment out of 3. The results are given as the mean +/- SEM (error bars) of the % of cells/ n°$RORC^{+}$ cells/µL in DSS treated animals (black bars) and untreated mice (white bars). Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test; ** $p < 0.01$; *** $P < 0.001$

These first findings suggest that wasting extinguishes monocyte production and promotes neutrophil expansion. Disrupted neutrophil/monocyte homeostasis correlates with BW loose, and a distinct expression pattern of molecular clock components in neutrophils and monocytes. A regain in BW and survival correlates with enhanced recruitment of monocytes and persistent Ci-sCG.

3.10. Neutrophils in cachexia exhibit a $Rorc1^{hi}$ signature

Strauss *et al.* have provided recent evidence of a novel immature myeloid cell population composed in 70-80% by $Rorc^{hi}$ (retinoic-acid-related orphan receptor (RORC1/RORc)) neutrophils that expands selectively in the spleen of animals with advanced tumor growth (Strauss et al., 2015a) an event which correlates with a 20% of body weight loss . Importantly, our recent data shows up-regulation of circadian clock transcription profile such as Npas2, Tef, Dbp, Per3 as well as IL-1-β, TNF-α and IL-10 in TAMs from mice with cancer AD when compared to TAM harvested from mice with early cancer ED (**Fig. 21**) This observation correlates with a body weight loss of ≥15% in mice with AD (Fig. 19) suggesting that circadian epigenetic control in myeloid cells from subjects with cancer cachexia may contribute to kinetics and function of myeloid cells.

RORC1 orchestrates "emergency" myelopoiesis by suppressing negative (Socs3 and Bcl3) and promoting positive (C/EBP-β granulopoiesis) in subjects with advance cancer, as well as the key transcriptional mediators of myeloid progenitor commitment and differentiation to the monocytic/macrophage lineage (IRF8 and PU.1). Further, RORC1 supports tumor-promoting innate immunity by protecting MDSCs from apoptosis, promoting MDSCs expansion in spleen, and limiting tumor infiltration by mature neutrophils (Strauss et al., 2015a).

We report in colitis cachexia neutrophil accumulation on expense of inflammatory monocytes. This event that correlates with up-regulation of TFs involved in cell metabolism to stress. NRs, including RORC1, have been recognized as a link between the circadian clock and metabolism (Yang et al., 2006) . Further, RORC1 has been identified as a factor, which controls adipogenesis as well as adipocyte size and

modulates insulin sensitivity in obesity (Meissburger et al., 2011). Collectively, these data point out RORC1 and related gene/transcription networks as plausible regulators of myeloid lineage commitment and function in inflammatory diseases that impinge on energy metabolism.

To analyse a plausible contribution of RORC1 to DSS-induced "emergency" myelopoiesis, we first determined RORC1 protein expression in $Ly6C^{hi}$ monocytes and $Ly6G^{+}Ly6C^{int}$ neutrophils in spleen and colon of mice with acute colitis at d7 (day of DSS withdrawal), at d9, in mice with colitis cachexia at d9 and mice in colitis remission at d14 (after 7d of DSS recovery).

As previously observed in advanced tumor growth (Strauss et al., 2015a), RORC1 expression in myeloid cells correlated with severe inflammation and BW loss (**Fig. 20**). A modest increase of $RORC^{+}$ $Ly6C^{hi}$ inflammatory monocytes was exclusively observed in colon from mice with cachexia (**Fig. 21**), and in acute inflammation in spleen (**Fig. 20**). An important increase of $RORC1^{+}Ly6G^{hi}$ neutrophils was identified in spleen and colon from mice with acute colitis at d9 (Fig. **20 and 21**). A dramatic increase in $RORC1^{+}$ neutrophil correlated with severe BW loss in colitis-induced- cachexia at d9 (>20% BWt loss; Fig. **20 and 21**).

RORC1 selective expression in neutrophils from mice with severe BW loss suggests a mediator role for nuclear orphan retinoic receptors in dysfunctional myeloid cell fate in cachexia.

RORC expression in macrophages was evident in all DSS treated groups (Fig. 25). Notably, there was an increase of $RORC^{+}$ $CD11c^{hi}$ $Class\text{-}II^{+}$ population at d9 cachectic and non- cachectic animals.

Intracellular staining for TNF-α revealed that >90% of $Ly6C^{hi}$ monocytes and $Ly6G^{hi}$ neutrophils from all study groups expressed TNF-α (data not shown). Equal cytokine protein expression levels between the study groups supports once more the hypothesis that disruption in clock molecular patterns in colitis cachexia is not directly due to inflammatory mediators.

3.11. Ci-sCG is disrupted in inflammatory neutrophils in colitis cachexia

Although the molecular clock is present in innate immune cells, its regulation and function in immune homeostasis remains unclear. Some inflammatory diseases may become chronic due to a failure of events that adapt the innate immune response to demand and initiate and control resolution of inflammation. Colitis is recently viewed as a dysregulated innate immune response with expression of gene/transcription networks that are involved in leukocyte activation and recruitment (Rahman et al., 2010). Our preliminary data shows that colitis cachexia correlates with reversion of Ci-sCC in colon and in splenic inflammatory $Ly6C^{hi}$ monocytes.

As we observed an increase of $RORC^{hi}$ neutrophils in subjects with cachexia, we wanted to investigate whether this phenomena might correlate with a characteristic clock gene signature as observed, for $Ly6C^{hi}$

monocytes in cachexia. Clock gene expression was determined in splenic $CD45^{+}CD11b^{+}Ly6C^{hi}Ly6G^{-}$ monocytes and $CD45^{+}CD11b^{+}Ly6G^{hi}Ly6C^{int}$ neutrophils (**Fig. 15B, Fig. 22**). Supporting our previous findings in colon, spleen and $Ly6C^{hi}$ inflammatory monocytes from mice with DSS induced colitis, expression of the core clock gene *Clock*, the PAR-bZip-CCG *Hlf*, and *Per3* was suppressed in splenic $Ly6G^{+}$ neutrophils, in comparison to neutrophils obtained from untreated mice at d9 of severe colitis (**Fig. 22**). Importantly, Ci-sCG was disrupted in $Ly6G^{+}$ neutrophils harvested from the spleen of cachectic mice, approaching the expression levels observed in splenic neutrophils from untreated controls (**Fig. 22**). Noteworthy, when comparing clock mRNA expression levels between neutrophils and $Ly6C^{hi}$ inflammatory monocytes, neutrophils from the different experimental groups showed significantly higher clock gene relative expression levels (**Fig. 22**). Interestingly, we also observed a significant increase in *Nampt* mRNA expression, the rate-limiting enzyme for NAD+ biosynthesis, in splenic neutrophils from cachectic mice (**Fig. 22**).

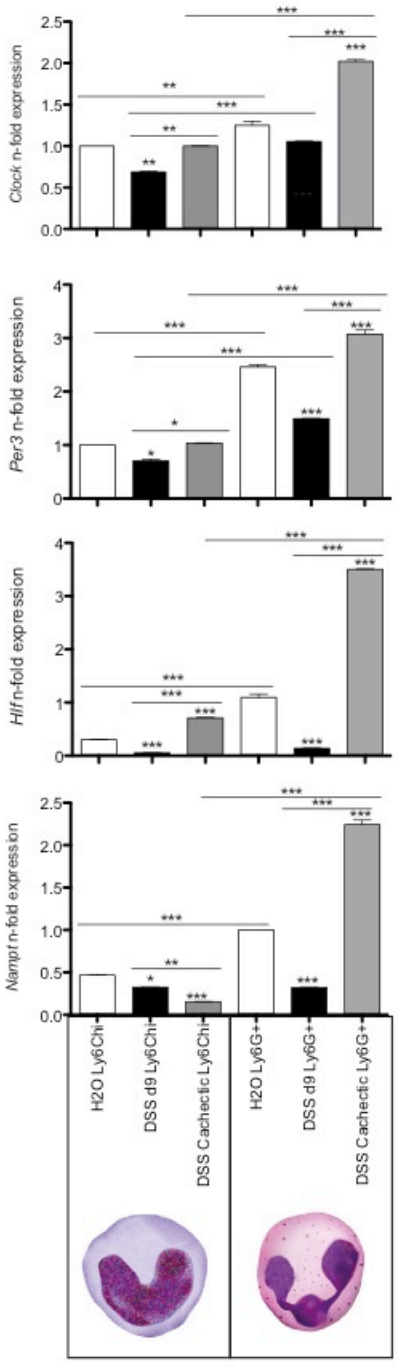


Figure 22. Ci-sCG reversion in neutrophils is associated with DSS-induced cachexia.

Splenocytes from DSS treated or H2O control mice were isolated at d9 or d14 and sorted using a FACS Aria III instrument (*See sorting strategy* **Fig. 15A**). FACS Dot plots show splenocytes from DSS treated mice. Debris (SSC-A vs. FSC-A, A) and doublets (FSC-H vs. FSC-A, B) were excluded and live/dead discrimination was determined using the amine reactive far red (SSC-A vs. L_D, C). $CD45^{+}$ $CD11b^{+}$ myeloid cells (D) were then sub-gated on $CD11b^{+}Ly6C^{+}$ cells (E), followed by excluding $Ly6G^{+}$cells (F). Inflammatory monocytes identified as$CD11b^{+}Ly6C^{hi}Ly6G^{-}$ (E, G), were FACs-sorted for gene analysis. Spleen from N≥8 mice /group was pooled prior to sorting. Afterwards, RNA was extracted from sorted populations and gene analysis by RT-qPCR was performed. Data shows mRNA relative expression of *Clock, Per3, Hlf* and *Nampt* in splenic neutrophils and monocytes from DSS treated mice, including cachectic mice at d9 (grey bars),

compared to untreated animals (white bars). The results are given as the mean of n-fold expression +/- SEM (error bars) of one experiment, comparing DSS treated mice with H2O control (open bars). Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test* $p<0.05$; ** $p < 0.01$; *** $P <0.001$

3.12. Colitis cachexia skews HSC differentiation towards RORC^{hi} GMP with disrupted Ci-sCG signature

To identify whether the monocyte/neutrophil dysbalance observed in cachexia reflects changes in mature cell populations or whether Rorc1^{hi} neutrophils with disrupted Ci-sCG signature may originate from dysregulated hematopoiesis, we investigated whether the upstream myeloid progenitor cells in BM were affected by intestinal inflammation/cachexia and if myeloid progenitors express Ci-sCG expression pattern. The changes observed in the cell populations observed in the periphery could be the result of modifications in proliferation of immature (lineage negative) myeloid progenitors or rather differences in the survival of mature cell populations, with only a modest contribution from the progenitor compartment. On the other hand, an uncontrolled increase of progenitor subsets could have considerable repercussions on the severity and chronicity of intestinal inflammation (Griseri et al., 2012).

Hematopoietic stem cells (HSCs) can self-renew and also give rise to the entire repertoire of hematopoietic cells. During acute infectious and inflammatory stresses, the hematopoietic system can quickly adapt to demand by increasing output of innate immune cells many-fold, often at the expense of lymphopoiesis and erythropoiesis. Hematopoietic stem cells (HSCs) give rise to multipotent progenitors (MPPs) that have lost self-renewal capacity and will later differentiate into highly proliferative lineage-committed progenitors: either common myeloerythroid progenitors (CMPs) or common lymphoid progenitors (CLPs) (Kondo et al., 2003). CMPs will segregate into either megakaryocyte-erythroid progenitors (MEPs) or granulocyte-monocyte progenitors (GMPs)(Akashi et al., 2000a). During colitis, the cell composition inside the myeloid progenitor (MP) population (Lin^{-}Sca-1^{-}c-Kit^{hi}) changed considerably. The percentage of GMPs among MPs increased from 27.3 ± 3.5 % to 53.9± 6% at d7 of acute colitis (**Fig. 23)** Noteworthy, post DSS withdrawal the % of GMPs increase to 61.1±5.2 % in non-cachectic mice at d9 and to 66.9±3% in cachectic mice at d9 (Fig.. 27), while the % of GMPs was equal to healthy controls in colitis remission at d14 (28.3±3.3%; Fig. 23C). The increase observed in the GMP population is mirrored by a substantial decrease in MEPs in the BM of colitic mice (29.2± 6.4% in H2O; 11.2± 3% d7; 7.8± 0.8 d9; 8.4± 1.4 cachectic ; 11.1± 2 d14; **Fig. 23**).

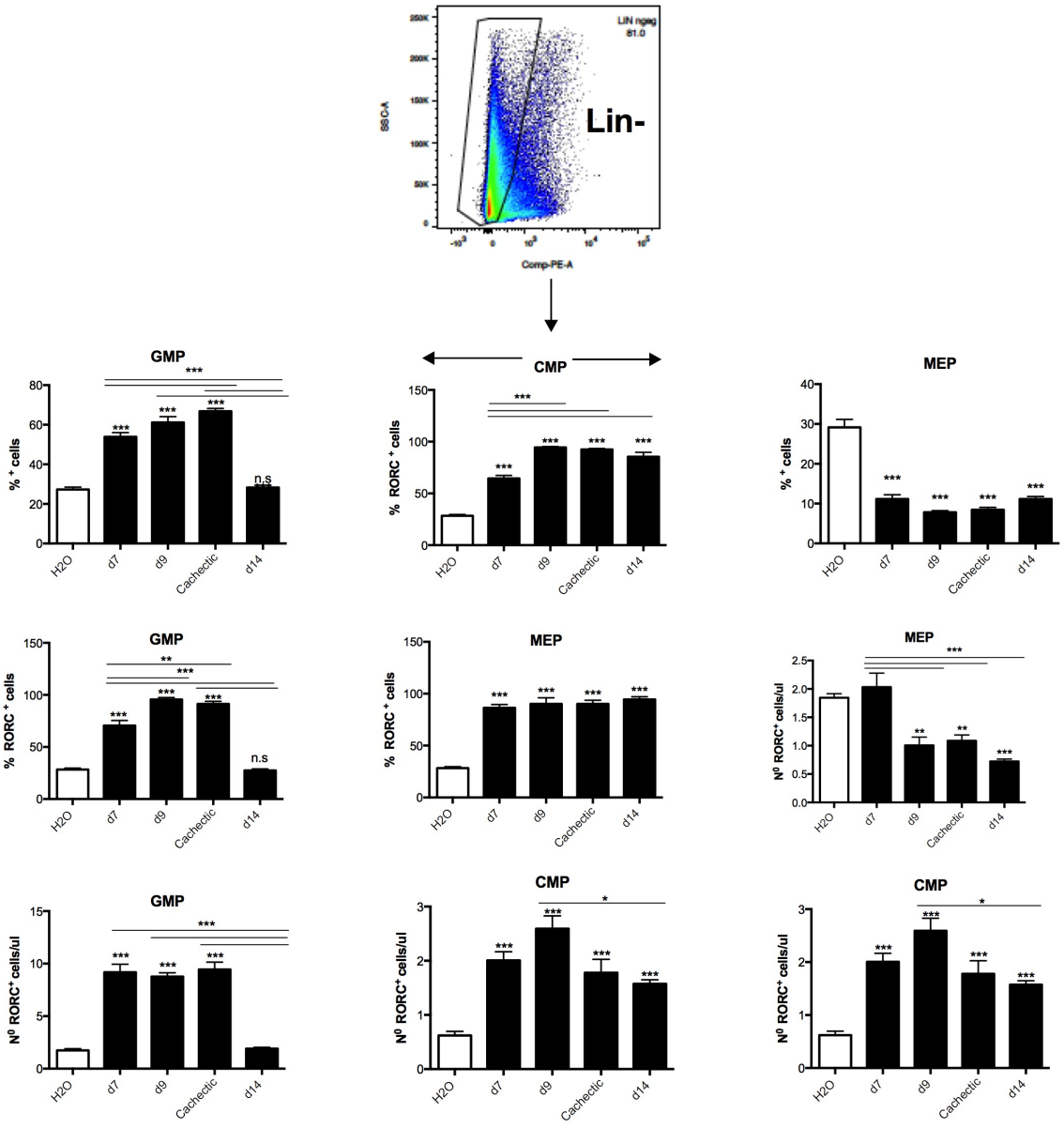


Figure 23. Bone marrow myeloid precursor dynamics in DSS-induced cachexia.

Bone marrow from WT and RORC-/- DSS-treated animals were taken from femur and tibia and mechanically processed to obtain single –cell suspensions, as described on experimental procedures. Mean +/- SEM (error bars) % and absolut (n°RORC$^+$ cells/µL) of GMP, CMP and MEP progenitors is shown. Data shows one representative experiment out of 3 with with n≥5 mice/ group. Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test; ** p < 0.01; *** P <0.001

Percentages of LSK (Lin$^-$ c-Kit$^+$ Sca1$^+$) and LK (Lin$^-$ c-Kit$^+$ Sca1$^-$) HSC populations and CMP were not significantly affected by colitis cachexia (data not shown). Further, the percentage of MDP in BM from mice with cachexia was not affected (data not shown), Expression of the chemokine receptor CX_3CR1 in GMPs (Akashi et al., 2000b) characterizes the MDP (Fogg et al., 2006) and is associated with the commitment of myeloid progenitors toward the macrophage/DC lineage. Thus, changes in myeloid lineage commitment to colitis cachexia appeared to be greatly limited to the granulocyte-monocyte progenitors.

IFN-γ is a well-known colitogenic cytokine that is increased in the colon and serum of colitic mice and its has a pathogenic role in colitis, not only by promoting effector functions in mature leukocytes, but also by boosting the hematopoietic activity at the level of the primordial HSCs that sustain the increased production of downstream and non-self-renewing GMPs (Griseri et al., 2012). Accordingly, we observed in all DSS treated groups increased IFN-γ in serum, although the difference were not significant between cachectic and non cachectic, and mice in remission (data not shown). Accordingly, we did not observed differences in LK/LSK percentages in DSS cachexia.

To investigate whether disrupted Ci-sCG in myeloid precursors may contribute to a dysbalance in systemic granulocyte-monocyte ratios, CMP and GMP were isolated from the BM of healthy controls, mice with acute colitis, colitis cachexia and colitis remission using FACS sorting (**Fig. 24**). Supporting our finding that Ci-sCG expression pattern is reverted in splenic neutrophils from cachectic mice, we found out that the Par-bZip-CCGs genes *Hlf* and *Tef* were also reverted in the granulocyte-monocyte progenitor GMP (**Fig. 25**). Noteworthy, Ci-sCG expression pattern was absent in CMP (Data not shown), evidencing so far that circadian epigenetic control in colitis is restricted to granulocyte/monocyte lineage commitment.

As Ci-sCG reversion in peripheral neutrophils correlates with RORC1 expression, we investigated RORC1 protein expression in GMP in BM of our different experimental groups using FACS analysis.

The % of RORC1 expressing GMP precursors increased in the BM of mice with acute colitis, peaking at d9 and in cachectic animals (**Fig. 23**), whereas RORC1 expression levels in the GMPs from mice in remission equaled the levels observed in healthy controls (**Fig. 23**).

We report here a novel granulocyte/monocyte lineage characterized by RORC1 expression and Ci-sCG reversion that arises selectively in animals that fail resolution of acute inflammation, event that leads to cachexia and ultimately death.

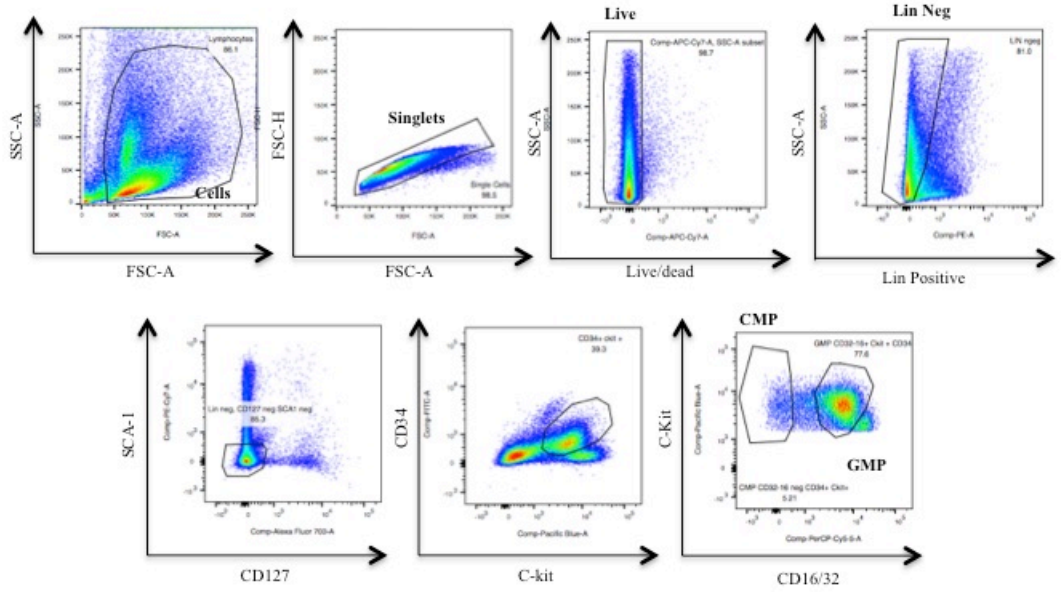


Figure 24. Sorting gating strategy of bone marrow GMP and CMP precursors.

BM hematopoietic cells from DSS treated mice (black bars) were isolated at d7, d9 (cachectic and non-cachectic mice (grey bars)), d14 and untreated mice, and sorted using a FACS Aria III instrument. Lin- population was enriched by negative selection of lineage positive cells prior to sorting, by using immune-magnetic columns. Debris (SSC-A vs. FSC-A, A) and doublets (FSC-H vs. FSC-A, B) were excluded and live/dead discrimination was determined using the amine reactive far red (SSC-A vs. L_D, C). Lineage negative cells (D) were then sub-gated on $CD127^-$ and $Sca\text{-}1^-$ (E), $ckit^+CD34^+$ (F),cells (followed by CD16/32 discrimination of CMPs ($CD16/32^-$) or GMPs ($CD16/CD32^+$), G).

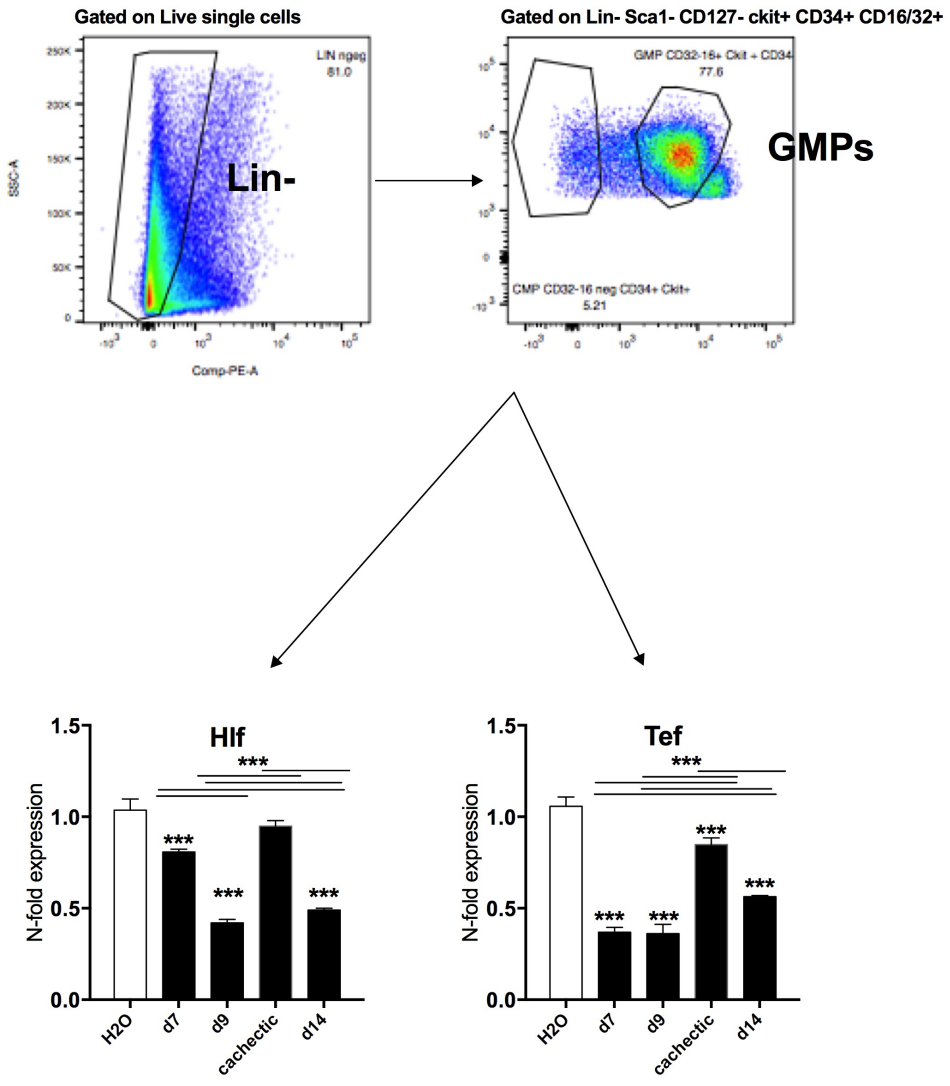


Figure 25. Ci-sCG reversion is observed in GMP myeloid precursors during DSS-associated cachexia

A. Sorting gating strategy of GMP precursors based on Lineage negative (lin^-) cells gated on (*See experimental procedures and sorting strategy,* **Fig. 24**).

B. BM from n≥8 mice /group was pooled and enriched for Lin^- cells prior to sorting. Afterwards, RNA was extracted from sorted populations and gene analysis by RT-qPCR was performed. Data shows mRNA relative expression of *Hlf* and *Tef* in hematopoietic GMPs from DSS treated mice, including cachectic mice at d9 (grey bars), compared to untreated animals (white bars). The results are given as the mean of n-fold expression +/- SEM (error bars) of one experiment, comparing DSS treated mice with H2O control (open bars). Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test; *** P <0.001

A key question, which remains unresolved, is what factors dictate polymorphonuclear versus mononuclear lineage fates during myelopoiesis. Strauss et al. recently reported that RORC expression is required for TAM maintenance, whereas depletion of $RORC^{hi}$ TAM led to an increase of tumor-infiltrating neutrophils in advance cancer disease, Furthermore, they showed that high expression of RORC in neutrophils led to accumulation of an heterogenous immature monocyte/granulocyte subset with a dominant neutrophil population in advanced cancer stages (Strauss et al., 2015a). This suggests that NRs may function as novel regulators of myeloid commitment to metabolic stress.

To further investigate possible factors, which might contribute to the differential myeloid population dynamics observed in acute colitis, ultimately leading to cachexia, we analyzed several cell fate decision TFs that are important at different stages of the myelopoiesis process. We first examined BM expression levels of *interferon regulatory factor 8 (Irf8*), a TF required for monocytic differentiation. Interestingly, we observed decreased *Irf8* mRNA expression levels in the BM from cachectic mice in comparison to healthy controls (**Fig. 26**), while its expression levels remained similar to H20 controls in the BM of mice in colitis remission. In contrast*, C/ebp-β*, a major driver of granulopoiesis, increased in all colitis study groups (**Fig. 26**). Noteworthy, inhibitors of granulopoiesis such as *Socs3* and *Bcl3* increased massively in the BM from mice in remission , but were hardly elevated in cachectic BM (**Fig. 26**). Previous studies reported that the IRF8 transcriptional activity is regulated by 12/15- lipoxygenase redox signaling and that the loss of its function, which impairs IRF8 nuclear translocation, leads to GMP accumulation (Kinder et al., 2010)

Collectively, these data show that RORC1 might induce immature progenitor and PMN-like-MDSCs accumulation in colitis, suggesting a new role of RORC1 promoting DSS cachexia. This event correlates with reversion of Ci-sCG in the myeloid precursor compartment.

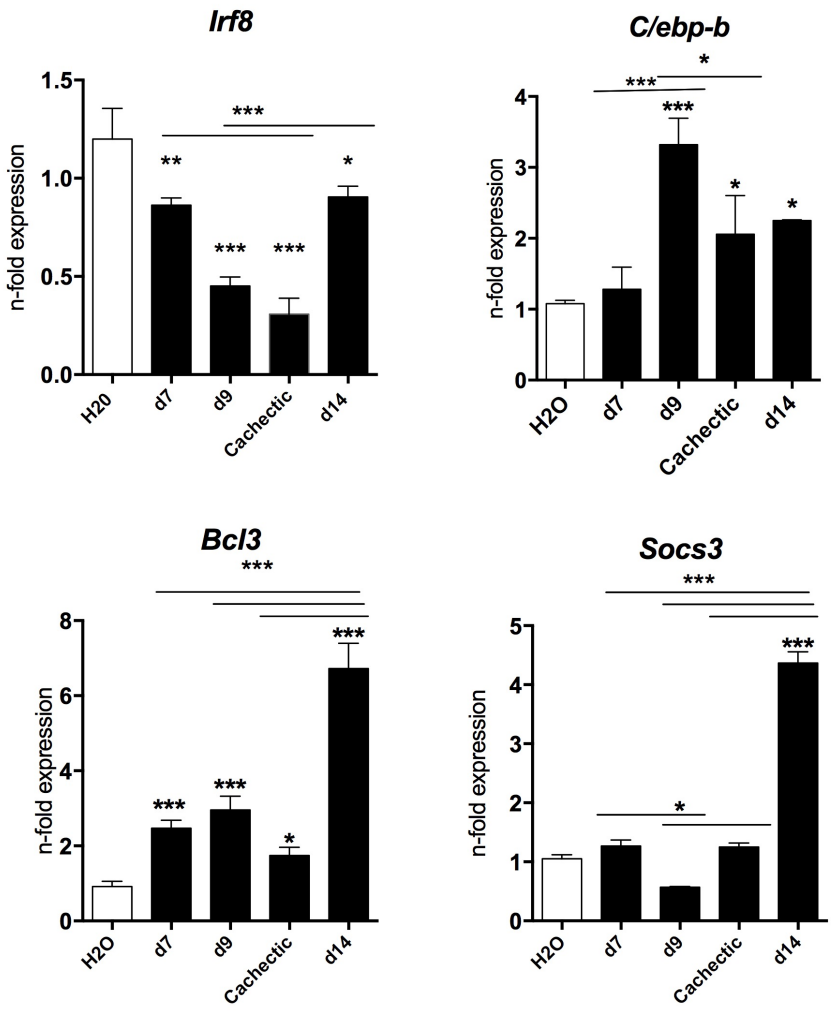


Figure 26. Bone marrow expression of 'emergency' myelopoietic factors.

Expression of *Irf8, C/ebp-β, Bcl3* and *Socs3* was analyzed in the BM of DSS treated at d7, d9 (including cachectic animals) and d14 (black bars), and in H2O controls. Data of RT-qPCR assays show one representative experiment out of 3 with n≥5 mice/ group. The results are given as the mean +/- SEM (error bars) relative expression (n-fold) of DSS treated mice, compared with their respective controls (open bars). Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test * $p < 0.05$; ** $p < 0.01$; *** $P < 0.001$

3.13. RORC1 -driven"emergency" myelopoiesis promotes cachexia

To assess the *in vivo* relevance of RORC1^{+} myeloid cells in development of resolution of inflammation in DSS colitis, we explored the effect of the RORC1 gene deletion. C57BL/6 WT mice and RORC$^{-/-}$ mice

were divided into two groups. One group of mice was sacrificed after 7 days of DSS treatment, while the other group of mice was allowed to recover from colitis until d14. No changes in food intake were noted throughout the duration of the study in any of the treatment groups (data not shown). Furthermore, core body temperture and locomotor activity was analysed in both groups during the 7d of DSS treatment by radiotelemetry i.p. sensors (*See Experimental procedures).*

Gut inflammation became evident from d5 of DSS treatment as assessed by BW loss and increased clinical score. Likewise WT animals, colitis progressed from d5 of DSS exposure. Signs of intestinal dysfunction (diarrhea and rectal bleeding) and BW loss peaked at d7 and d9, respectively (**Fig. 27 A-C**).
However, DSS-challenged RORC -/- mice lost less than 20% of their original weight at d9 post DSS treatment (**Fig. 27A,B**). Noteworthy, while 10-15% of WT mice develop severe body weight loss (>20%) and a more severe clinical score at d9 (**Fig. 27C**), events that both correlate with an increased drop-out rate after DSS removal, RORC -/- animals do not develop cachexia. Furthermore, re-gain of BW post disease peak was reached faster (**Fig. 27A,B**).

Clinical features of IBD, including diarrhea and presence of rectal bleeding, were also monitored and scored. Colitis clinical features were significantly worse in DSS-challenged WT than in KO mice from d5 onwards (**Fig. 27C**). Similarly, DSS-challenged WT mice exhibited more severe histologic score than RORc-/- animals in both leukocyte infiltration and epithelial damage at all studied timepoints (**Fig. 27D**).

Thus, the lower clinical and histopathologic scores, together with the reduced BW loss in DSS-challenged KO, are indicative of the development of less severe colitis in RORC deficint mice.

A reduction in the length of the colon is used as an indirect measurement of inflammation and it was shown to be directly proportional to the severity of experimental colitis and in patients (Xu et al., 2008). However, while all DSS-challenged mice groups had shorter colons that the respective controls, there was no difference in length between WT and RORC depleted animals **(Fig. 27E).** Nevertheless, differences in spleen weight were evident. Mainly, the increased was observed at d14, in colitis remission (**Fig. 27F**).

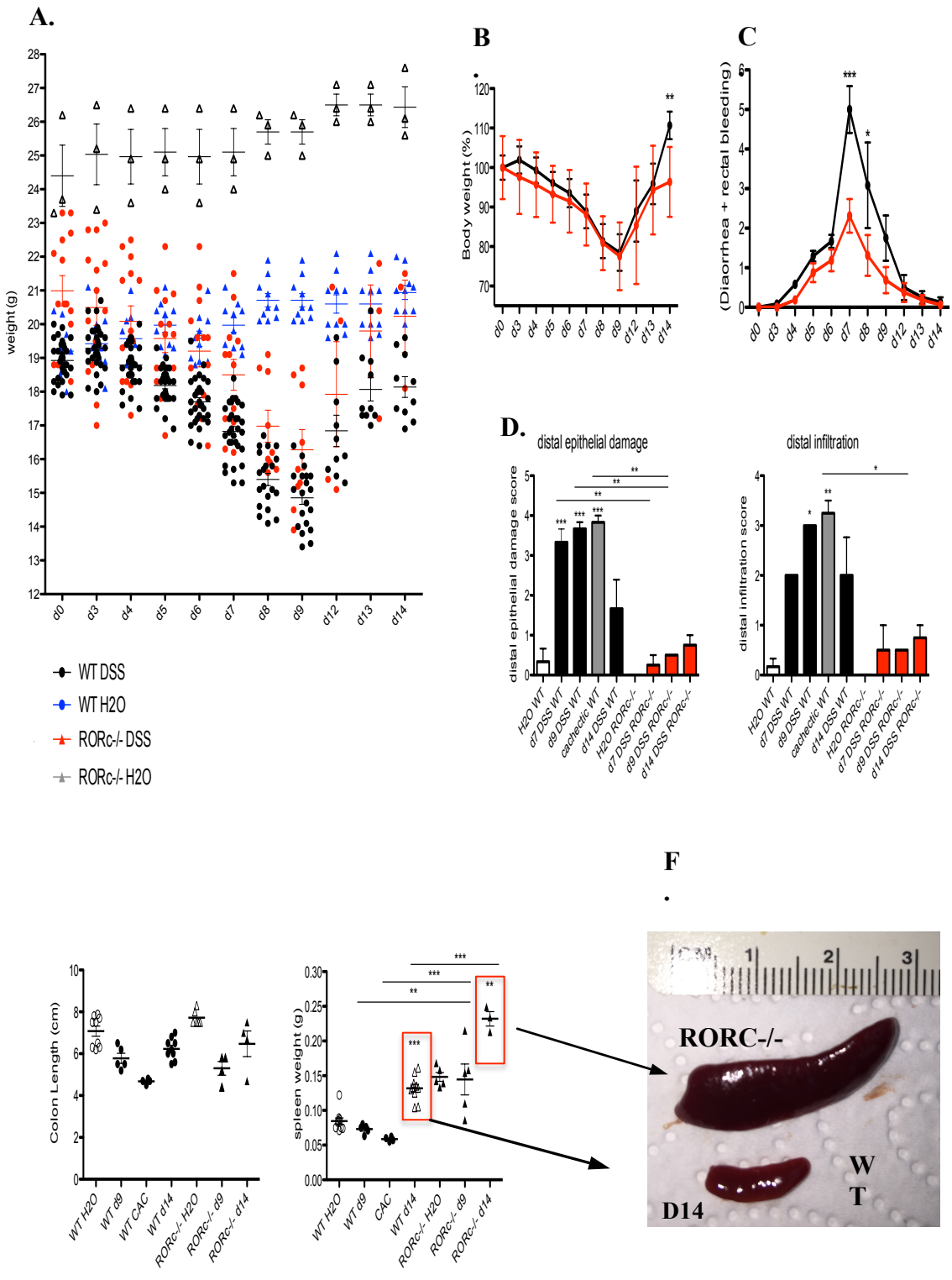


Figure 27. RORC deficient mice are protected from DSS-associated cachexia.

DSS acute colitis was induced in WT and RORC -/- animals for 7d, followed by 7d with normal drinking water. Animals were monitored daily to evaluate clinical parameters of colitis: Absolut BW (g, A.), BW

loss (%, B.), Colitis score (diarrhea and rectal bleeding, C.), histopathogical score (intestinal epithelial damage and leukocyte colonic infiltration, D.), colon length (cm) and spleen weight (g) (E.). In colitis remission at d14 both WT and RORC deficient showed increased spleen weight. WT vs RORC -/- at d14 (F.). The results are given as the mean +/- SEM (error bars). Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test * p < 0.05**; p < 0.01; *** P <0.001

As reported previously ROR -/- mice have increased BW, compared to WT animals of the same age and sex, due to alterations in adipocyte size distribution (Meissburger et al., 2011), indicating that RORC depletion might lead to a slow down in energy metabolism. Furthermore, RORC -/- animals are protected from diet-induced-obesity. This suggests that a decreased energy metabolism might delay disease progression, which could be in part mediated by upstream regulators of myeloid lineage commitment.

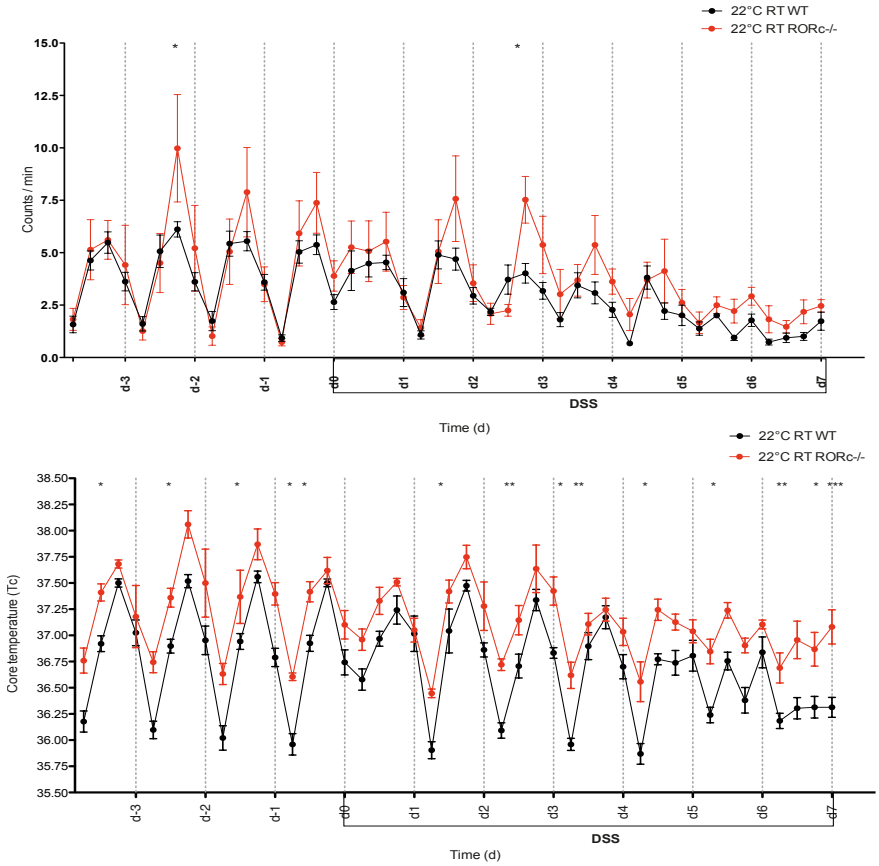


Figure 28. Locomotor activity and body core temperature in RORC -/- with acute colitis.
Locomotor activity was assessed by implanting intraperitoneal (i.p) telemeters (TA-F10, DSI) in WT and RORC -/- mice mice under isofluorane anesthesia, two weeks prior to acute colitis induction. Telemetry recordings were based on mean average counts per minute (cpm) of 1 h intervals by using the Dataquest A.R.T. software. Activity and temperature measurements were performed continuously during the whole course of the experiment from d-3 to d7 of DSS treatment. Two-way ANOVA for repeated measurements. Bonferroni posthoc test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

However, when assessing locomotor activity and body core temperature by telemetry, we observed that both parameters were higher in the RORc -/- during their active phase, even before DSS treatment (**Fig. 28**). This indicates that behavior as assessed by locomotor activity is less affected in RORC -/- mice compared to WT. Increased locomotor activity is associated with increased body temperature.

3.14. RORC1 expression is needed for "emergency" granulopoiesis in DSS-induced colitis

Strauss et al. have reported previously that RORC1 expression in the hematopoietic compartment is required for "emergency" myelopoiesis in advanced tumor bearing mice, whereas steady state myelopoiesis in healthy animals is RORC1 independent (Strauss et al., 2015a). Nevertheless, the role of RORC1 in directing myelopoiesis in acute inflammatory disease and in inflammation resolution has to date not been investigated. Here we report accumulation of GMP and a systemic neutrophil/monocyte ratio skewed to neutrophils in cachectic mice that fail resolution of inflammation. We evidence for the first time that GMP and neutrophils in cachexia express a RORC1 signature. Importantly, we demonstrate that RORC1 deficiency as well as its selective depletion in the myeloid compartment (data not shown) smolders severe colitis and prevents from colitis-associated cachexia (*See introduction,* **Fig. 7***)*.

As a next step, we examined the process of "emergency" myelopoiesis after DSS exposure in C57BL/6 WT mice and RORC -/- mice. Firstly, we compared the kinetics of $Ly6C^{hi}$ monocytes and $Ly6G^{+}$ $Ly6C^{int}$neutrophils in spleen and colon of mice with acute colitis at d9, mice with colitis cachexia at d9 and mice in colitis remission at d14. As shown in **Fig. 27** a less severe colitis, less BW loss and protection from cachexia was found to correlate with a massive decrease of $Ly6G^{hi}Ly6C^{int}$ neutrophils at d9 in spleen and colon from RORC -/- mice, compared to the WT (**Fig. 29,30**).

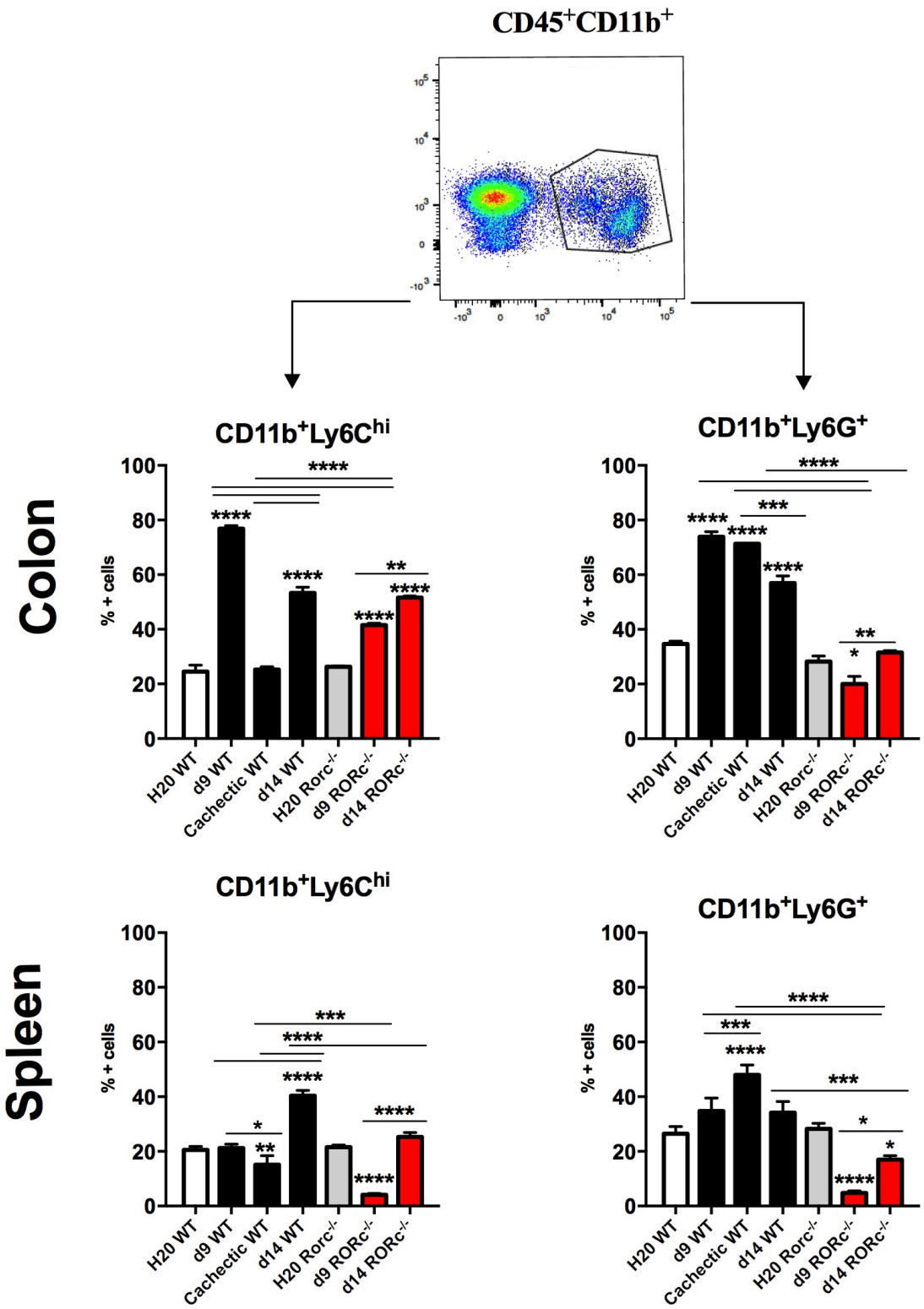


Figure 29. Myeloid spleen and colon subsets in RORC -/- mice.

Splenic and LP colonic myeloid cells were isolated and analyzed by FACS, as described in experimental procedures and in figure 23 (gating strategy). Dot plots show one representative experiment out of 3. DSS treated animals (black bars) and untreated mice (white bars). The results are given as the mean +/- SEM (error bars). Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test * $p < 0.05$;** $p < 0.01$; *** $P < 0.001$

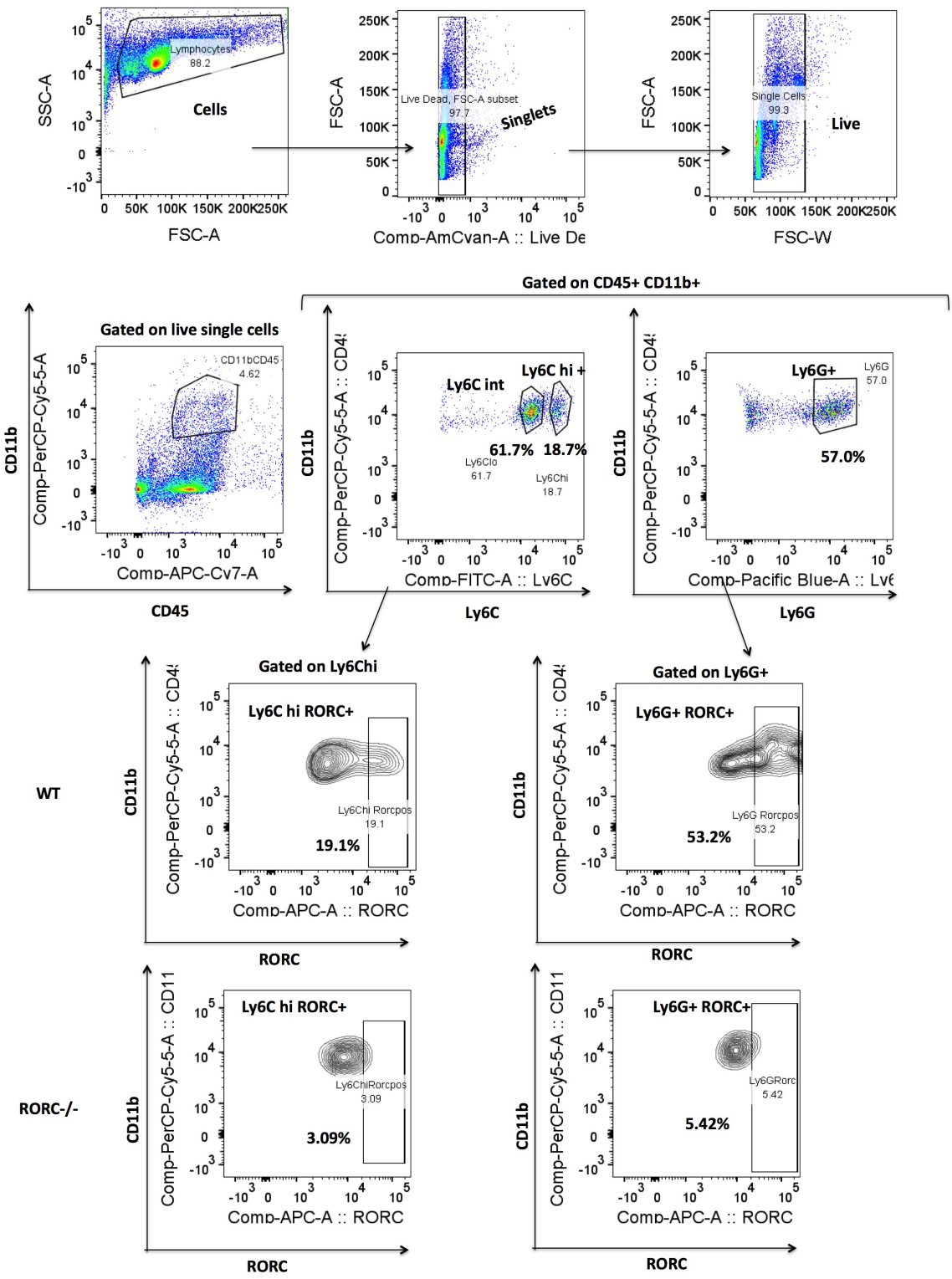


Figure 30. Gating strategy for spleen myeloid subsets and RORC expression in WT and RORC deficient mice.

Splenocytes were isolated at d7, d9 (cachectic and non-cachectic) and d14 from DSS treated and H2O control mice and phenotypically analyzed by FACS Aria III instrument. Debris (SSC-A vs. FSC-A) and doublets (FSC-H vs. FSC-A) were excluded and live/dead discrimination was determined using the amine

reactive far red (SSC-A vs. L_D). $CD45^+$ $CD11b^+$ myeloid cells were then sub-gated on $CD11b^+Ly6C^+$ cells and $Ly6G^+$cells. Inflammatory monocytes were identified as $CD11b^+Ly6C^{hi}Ly6G^-$ and neutrophils as $CD11b^+Ly6G^+Ly6C^{int}$.The above-described populations were analyzed for the expression on RORC (% of $RORC^+$ cells is shown) in WT and KO animals.

Nevertheless, RORC -/- showed increased monocyte recruitment in the colon from d9 onwards, until the end of the experiment at d14, although the monocytes levels at d9 were remarkably lower than in the WT non-cachectic animals (**Fig. 29**). However, as mentioned previously, cachectic WT mice failed to recruit inflammatory monocytes in colon. This holds also true when analyzing the spleen. However, there the levels did not increase significantly until d14, which correlated with increase spleen size)). Of note, RORC -/- spleen size was significantly bigger than in their WT littermates, including in baseline conditions. Remarkably, spleen weight in RORC deficient mice increased dramatically at d14, compared to WT animals (**Fig. 27E).** These findings support our hypothesis that $RORC^+$ neutrophils play an essential role in promotion of the inflammatory response as well as in the resolution machinery.

3.15. RORC depletion expands MEPs, mirrored by a substantial decrease in GMPs

To identify whether RORC expression in myeloid progenitors was needed for GMP accumulation mirrored by a decrease in MEP expansion in colitis, BM from WT and RORC -/- mice were analyzed for CMP, GMP and MEP composition inside the myeloid progenitor population (MP, $Lin^-Sca\text{-}1^-c\text{-}Kit^{hi}$) (**Fig. 32**). The percentage of GMPs among MPs at d7 of acute colitis in RORc deficient mice when compared to WT animals was similar (53.9±6% WT and 57±2.3% KO; **Fig. 31**). Noteworthy, post DSS withdrawal the % of GMPs continue increasing in WT non-cachectic at d9 and cachectic mice (62±65.2 and 66.9±3% , respectively). This seems less pronounced in RORC -/- animals, were no significant differences between d7 and d9 were observed (53.4±11.6% ; **Fig. 31**).

On the other hand, CMP % were decreased in all DSS treated groups in comparison with the untreated controls in the RORc -/-, in contrast to their WT counterparts, which showed similar levels than H2O controls, with the exception of d14, when they showed a significant increase in CMPs (7.8±1.4% H2O KO vs 8.8±2% H2O WT; 1.4±1.1% d7 KO vs 11.1±1.6% d7 WT; 5±1.9% d9 KO vs 16.13±2.9% WT d9 non cachectic and 11.3±2.8% WT cahectic; 4.4±2.3% d14 KO vs 22.3±6.4% d14 WT; **Fig. 31**). Interestingly, and in contrast to WT animals, the substantial decrease in CMPs in the RORC -/- was mirrored by an increase in MEPs, remarkably at d14 (19.6±5.4% H2O KO vs 29.2±6.4% H2O WT; 16.8±1.2% d7 KO vs 11.2±3% d7 WT; 11±4.2% d9 KO vs 7.8±0.8% WT d9 non cachectic and 8.4±1.4% WT cachectic; 29.5±6.8% d14 KO vs 11.1±2% d14 WT; Fig. 35). Further, the percentage of MDP in BM from RORC -/- mice with colitis was not affected when compared to WT mice (data not shown).

These findings evidence that RORC expression in myeloid progenitors drives GMP accumulation mirrored by a substantial decrease in MEP in mice with severe colitis and colitis cachexia, events that correlates with decreased survival.

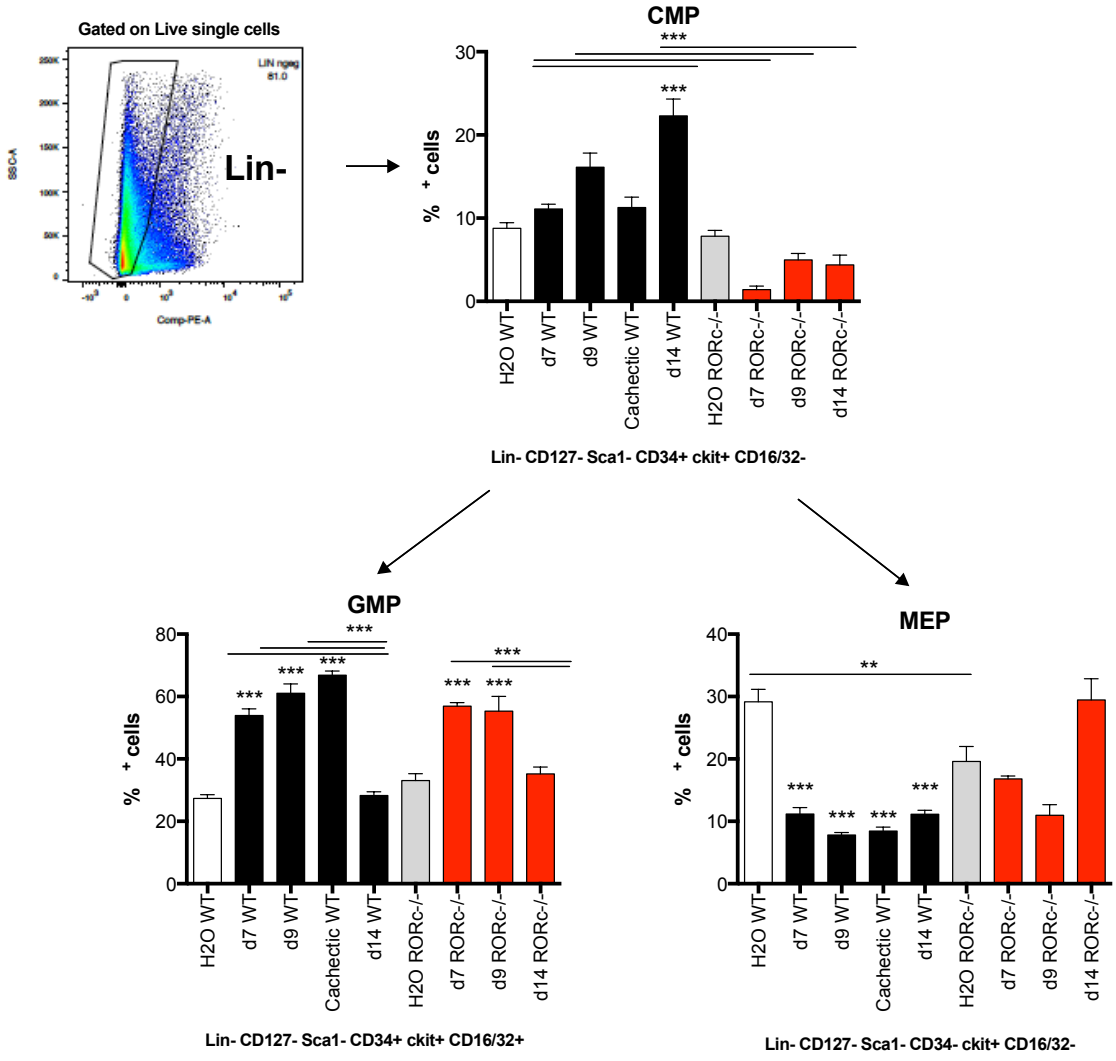


Figure 31. BM hematopoietic precursor populations in RORC -/- with acute colitis.

Myeloid BM precursors myeloid cells were isolated from DSS treated RORC -/- mice and WT and analyzed by FACS, as described in experimental procedures and in figure 36 (gating strategy). Dot plots show one representative experiment out of 3. DSS treated animals (black bars) and untreated mice (white bars). The results are given as the mean +/- SEM (error bars). Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test; ** $p < 0.01$; *** $P < 0.001$

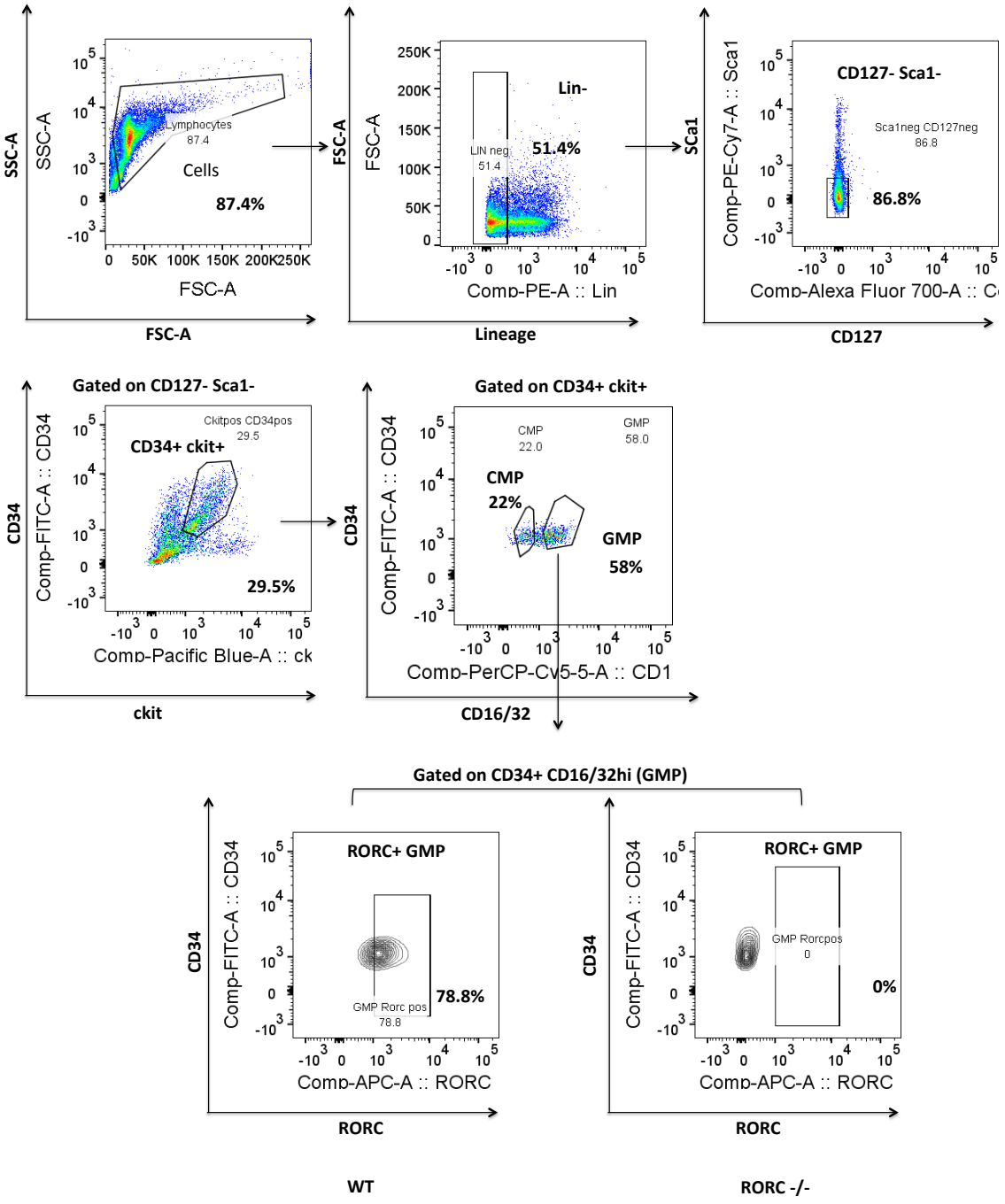


Figure 32. Gating strategy for hematopoietic bone marrow subsets and RORC expression in WT and RORC deficient mice.

BM hematopoietic cells from WT and RORC -/- mice DSS treated were isolated at d7, d9 (cachectic and non-cachectic mice), d14 and untreated mice, and sorted using a FACS Aria III instrument. FACS Dot plots form a representative experiment out of 3 is presented here. Debris (SSC-A vs. FSC-A, A) and doublets (FSC-H vs. FSC-A, B) were excluded and live/dead discrimination was determined using the amine reactive far red (SSC-A vs. L_D, C). Lineage negative cells $CD45^+$ $CD11b^+$ myeloid cells (D) were then

sub-gated on CD127$^-$, Sca-1$^-$, ckit$^+$, CD34$^+$ cells (followed by CD16/32 discrimination of CMPs (CD16/32$^-$) or GMPs (CD16/CD32$^+$)) or CD34$^-$ cells (MEPs). The above-described populations were analyzed for the expression on RORC (% of RORC$^+$ cells is shown).

3.16. RORC promotes induction of the G-CSF/CXCL12 axis and Ci-sCG reversion in colitis

Having observed a difference in the course of disease with increased resolution of inflammation and protection from cachexia in RORC -/- mice, we investigated whether this was reflected and possibly attributable to a smoldering effect of RORC depletion on changes in mediators of emergency myelopiesis or circadian epigenetic control.

RT-qPCR analysis of spleen segments showed a dramatic decrease in *G-csf* and *Cxcl-12* in RORC -/- mice with colitis when compared to WT mice at d9/cachexia (**Fig. 37**). Accordingly to down-modulation of pathways that predispose to cachexia, circadian TFs involved in epigenetic control to metabolic stress such as *Npas2*, *Per3*, *Hlf* were significantly down-regulated in spleen from RORC -/-animals at d9 when compared to cachectic WT mice at d9. Similarly, their expression levels remained decreased at d14 of disease resolution (**Fig. 33**). Importantly, also *Nampt* expression levels, the rate-limiting enzyme in the NAD salvage pathway (Zhang et al., 2011), were significantly down-regulated in spleen from RORC-/- compared to their WT counterparts , where we observed increased *Nampt* levels in cachectic mice, in contrast to non-cachectic animals (**Fig. 33**).

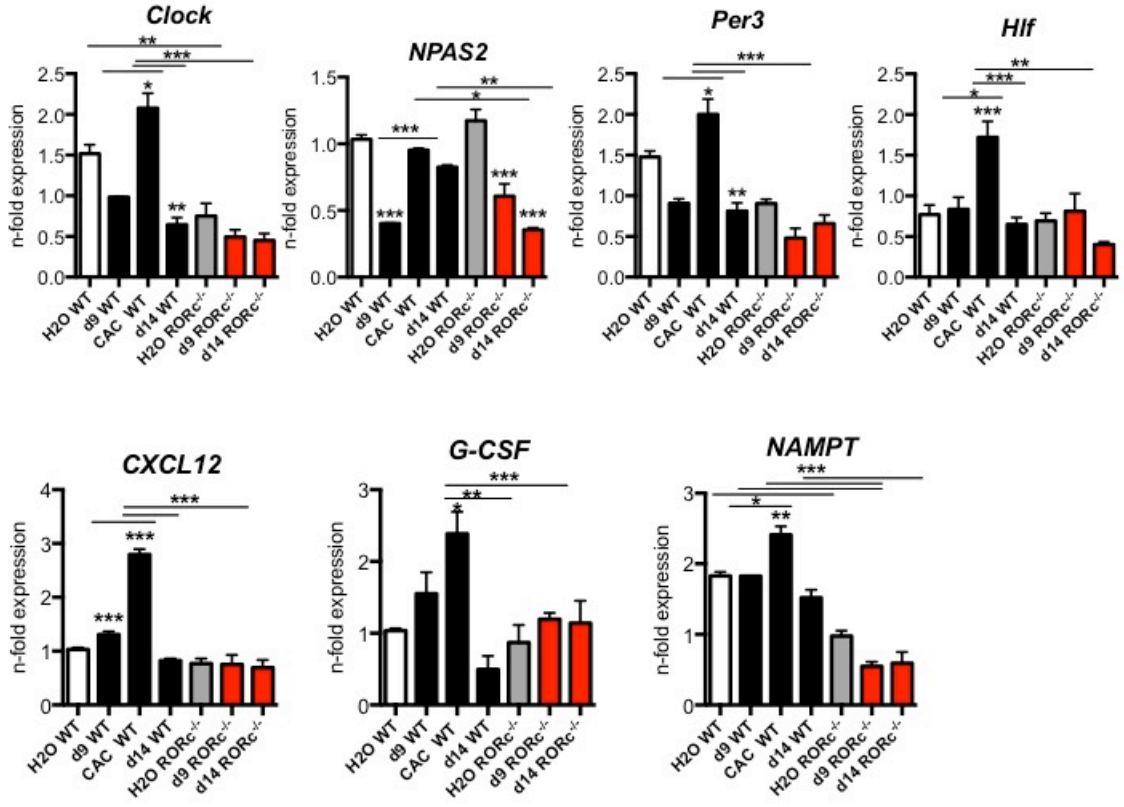


Figure 33. Gene expression in RORC -/- with acute colitis.

Expression mRNA levels of *Clock, Npas2, Per3, Hlf* , *Cxcl12*, *G-csf* and *Nampt* in WT DSS treated mice (black bars) and untreated controls (white bars) and RORC -/- DSS treated animals (red bars), and their respective untreated controls (grey bars) were determined by RT-qPCR. The results show one representative experiment out of 3 n≥5 mice/ group. The values are given as the mean +/- SEM (error bars) comparing triplicates of DSS treated mice with H2O control (open bars). Significance of grouped results were calculated with one-way-ANOVA and Bonferroni posthoc test * $p < 0.01$; ** $p < 0.01$; *** $P < 0.001$

Taken together, our data suggests that a disruption in epigenetic clock control of "emergency" myelopoiesis to severe or persistent inflammation leads to accumulation of RORC1 monocyte/neutrophil progenitors in BM and $RORC^{+}$ PMN-like-MDSC in spleen, while an important decrease in $Ly6C^{hi}$ monocytes in spleen is observed. Importantly, mice with colitis remission do not accumulate $RORC^{+}$ GMPs in BM and exhibit an important increase of $RORC^{lo}$ $Ly6C^{hi}$ monocytes in spleen. These preliminary analyses suggest a potential novel link between RORC mediated "emergency" granulopoiesis and metabolic stress.

Chapter 4.

DISCUSSION

Chapter 4. DISCUSSION

Ci-sCG reversion is associated to DSS-induced cachexia

The clock machinery and immune system communicate with each other through tightly regulated feedback loops. The molecular clock influences the extent of immune effector mechanisms (Curtis et al., 2014). On the other hand, activation of the immune system leads to cytokine mediated interference of clock genes (Cavadini et al., 2007). Here we report for the first time that in DSS- induced colitis the expression of the clock genes *Clock, Per3, Dbp, Tef* and Hlf, is reduced in total colon and spleen, as well as in sorted splenic inflammatory monocytes and neutrophils, in comparison to healthy controls. This observation is in line with a significant rise in the expression of the proinflammatory cytokines TNF-α and IL-1-β, supporting our previous observations that the aforementioned genes are suppressed in the liver of mice injected with TNF-α or anti-CD40 antibodies, the effect being associated with increased sleep and development of depressive like behaviour (Cathomas et al., 2015; Taraborrelli et al., 2011). Intriguingly, the inflammation associated inhibition of *Clock, Per3, Dbp, Tef* and *Hlf* (Ci-sCG)) is not only a hallmark of acute colitis, but is extended to colitis remission, characterized by a recovery of BW loss, despite a significant increase of proinflammatory cytokines, in comparison to the respective controls. This hypothesis is supported by the observation that Ci-sCG is reverted to mRNA expression levels of healthy controls, in colon and spleen as well as in inflammatory monocytes and neutrophils in mice that fail to recover from acute colitis after DSS removal. Ci-sCG reversion and disease remission failure is associated with cachexia and lethal outcome. These findings suggest that Ci-sCG may constitute an essential component in resolution of inflammation and that might be caused by other mechanisms than pro-inflammatory cytokines.

Circadian clock genes have been shown to regulate the physiology and function of the GI tract. Disruption of the circadian clock (e.g., shift workers) has been linked to gastrointestinal disease, including exacerbated irritable bowel syndrome symptoms and increased risk for developing colorectal cancer (Nojkov et al., 2010; Schernhammer et al., 2003). Furthermore, recent studies demonstrated that chronic circadian disruption using mice homozygous for the $Clock^{\Delta lo}$ mutation, worsened experimental colitis course. Repeated phase shifts of the light:dark (LD) cycle accelerated disease onset, worsened severity of histopathological damage, exacerbated inflammation and increased mortality (Preuss et al., 2008).

DSS-associated-cachexia exhibits a catabolic signature

In recent transcriptome analysis focused on the analysis of clock genes in human colon biopsies, *Arntl2* and *Rora* were up-regulated, while *Csnk2b, Npas2, Per1* and Per3 were down-regulated in Crohn`s disease. This contrasted the results reported in ulcerative colitis, which showed *Arnlt2, Cry1, Csnk1E, Rora* and *Tipin* up-regulated, while *Nr1d2* and *Per3* were down-regulated (Palmieri et al., 2015). These preliminary

findings support our results in murine colitis, showing that differences in severity and outcome of colitis are associated with divergent clock gene expression patterns.

To date the role of the molecular clock in mediating gut inflammation and disease remission has not been addressed. Clock genes have been reported to be necessary in host immune response regulation, including cytokine expression (Curtis et al., 2014) . About 8% of the genes expressed in peritoneal macrophages show circadian regulation (Keller et al., 2009). BM-derived-macrophages from *Clock* mutant mice treated with LPS express significantly less TNF-α and IL-1-β, indicating that the molecular clock supports proinflammatory cytokine production in innate immune cells (Bellet et al., 2013). The molecular clock may modulate innate immunity and contribute to the course of gut inflammation. Thus, disrupted clock gene expression in DSS induced colitis might be associated with the amplitude of the inflammatory response. Nevertheless, severity markers such as colon length and histological score were not significantly different in mice with DSS induced cachexia and non-cachectic animals, despite their differential clock gene expression. Accordingly, proinflammatory cytokine mRNA expression levels in colon between the two analyzed experimental groups (cachectic and non cachectic mice) and locomotor activity did not differ either. Thus, Ci-sCG seems unlikely to modulate the amplitude of the primary cytokine-mediated-inflammatory gut response in mice with DSS-induced-colitis. The data presented here associates resolution of gut inflammation with persistent Ci-sCG.

The finding that cachectic mice with a loss of ≥ 20% BW showed a reversion of Ci-sCG might indicate that differential clock gene expression pattern in colon and spleen may be rather involved in control of energy metabolism to inflammatory stress. These observations suggest that a balanced metabolic response may be crucial in mediating the amplitude and resolution of the inflammatory response.

Cachexia is the major contributor to morbidity and premature mortality from cancer and many other chronic diseases (Fearon et al., 2013). The weight loss associated with cachexia results from a reduction of lean mass, which is composed mostly of skeletal muscle (Evans et al., 2008). Patients with IBD suffer from BW loss, malnutrition and other metabolic alterations affecting their quality of life. Calorie restriction has been associated to a low inflammatory status (Ye J., Ageing 2010). Melgar et al. reported that acute and chronic DSS experimental colitis, similar to IBD patients, is associated with significant metabolic alterations, characterized by decreased metabolic rate. The authors showed that mice exhibited decreased fat and lean mass some days after initiating DSS treatment, and recovery from DSS colitis was associated with increased food/water intake, lean mass and respiratory exchange ratios (Melgar et al., 2007).

In our experiments, mice with acute colitis showed reduced glycaemia at the peak of the disease with a return to control levels during disease remission. Despite of a presumably increased catabolism in DSS-Cachectic animals, they did not turn to be more hypoglycemic than DSS treated animals with a decreased BW loss.

Dong and colleagues recently characterized metabolic signatures of DSS-induced acute colitis from urine and plasma of mice, which showed that metabolites associated with gut microbiota, oxidative stress, and regulation of energy levels, were altered by DSS insult (Dong et al., 2013). They reported a decrease in BW

gain, food intake, as well as reduced level of plasma and liver glucose and anemia in DSS acute colitis. The authors proposed that the damage induced by DSS-treatment to the architecture of the colon mucosa could lead to compromised colon function (such as absorption) and subsequently, BW loss and anemia. Besides, they observed a raised urinary level of TCA cycle intermediates, implying that the stimulated glycolysis facilitated the rate of the TCA cycle. Finally, they detected as well increased amount of aminoacids in plasma, reflecting protein catabolism. Hence, suggesting the existence of coordinated changes in energy metabolism, reflected by an increased energy consumption (Dong et al., 2013).

Recently, several studies have recognized the importance of lipid metabolism and TAG hydrolysis as a major metabolic pathway involved in the initiation and progression of cachexia (Das and Hoefler, 2013). Several groups have reported that severe inflammation shifts lipid metabolism to a catabolic state, thus decreasing the fat mass in cachectic patients (Petruzzelli et al., 2014). However, the impact of this catabolic shift on the systemic immune response has not been determined to date. The data shown here link the absence of Ci-sCG to catabolic signaling and progressive BW loss in inflammation-associated cachexia. Inflammation-associated cachexia leads to a complete breakdown of adipose tissue and skeletal muscle, including whole body lipolysis, proteolysis and glycolysis in severe cachexia. Loss of body mass is due to increased metabolic catabolism by host products, including the primary site of inflammation (Tisdale, 2002). Ci-sCG might play a pivotal role in resolution of colitis, preventing metabolic dysbalance and harmful inflammation. Our novel findings suggest that metabolic cues associated to severe inflammation, may modulate circadian epigenetic signatures in splenic monocytes and neutrophils that might control their effector capacity, plasticity and life span and therefore influence the fate of the inflammatory response and outcome.

Cachexia as a hypermetabolic syndrome and its link with disrupted circadian clockwork

Circadian oscillators in peripheral tissues can participate in the control of rhythmic metabolism through circadian TFs, which in turn regulate the cyclic transcription of metabolically relevant downstream genes. Genome-wide transcriptome profiling of WT and PAR bZip triple KO mice has revealed differentially expressed genes involved in lipid metabolism, many of which are targets of NRs, like PPARα (Gachon et al., 2011) . Furthermore, biochemical work by McKnight and coworkers on NPAS2 and CLOCK suggests that circadian rhythms could be influenced directly by cellular metabolism (Rutter et al., 2002). At least in vitro, the dimerization of NPAS2 or CLOCK with BMAL1 and/or the binding of the resulting heterodimers to their DNA recognition sequences is dramatically modulated by the ratio of $(NAD(P)^{+}$ and NAD(P)H. Suggesting the possibility that circadian oscillators may adapt their phase to the nutrient state of the cell (Gachon et al., 2004).

Accumulating evidence suggests that disruption of circadian rhythms is closely associated with metabolic syndrome (Maury et al., 2010). Bmal1 and Clock have been implicated in the regulation of glucose

metabolism and dysfunction of these transcription factors leads to glucose intolerance (Rudic et al., 2004; Zhang and Kay, 2010). Mouse models of genetically manipulated clock components Bmal1 or Clock display features of metabolic syndrome-like phenotypes comprising obesity and insulin-resistance (Gomez-Abellan et al., 2008) or leanness (Froy, 2011), respectively.

Circadian oscillators in peripheral tissues can participate in the control of rhythmic metabolism through circadian TFs, which in turn regulate the cyclic transcription of metabolically relevant downstream genes. The three PAR-bZip proteins, DBP, HLF and TEF, have regulator roles in cellular processes such as nutrient utilization, cell development and various stress responses (Kong et al., 2015) , including xenobiotic/oxidative-stress induced apoptosis (Ritchie et al., 2009).

Previous studies have found that loss of adipose tissue and muscle mass in cachexia is mediated predominantly by increased lipolysis and proteolysis associated with a hypermetabolic state (Das et al., 2011; Rydén et al., 2008). During cachexia, a prevalent catabolic state leads to energy inefficiency and increase of oxidative stress, with consequent exhaustion of fat reservoirs and muscle growth impairment. Oxidation reactions are crucial for metabolism and signal transduction. However, they generate ROS, which damage lipids, proteins and DNA, generating oxidation-specific epitopes or DAMPS (Miller et al., 2011) that can be sensed not only through DRRs but also through PRRs in hematopoietic and non hematopoietic cells, leading to a stress-induced-myelopoietic response.

Furthermore, oxidized metabolites can be sensed through nuclear receptors such RORC, with the potential of promoting the expansion of suppressor myeloid populations, leading to a failure in resolution of inflammation and tissue repair, as well as potentiate secondary infections, contributing to poor survival.
The differential expression of clock output mediators observed in DSS cachexia might indicate the existence of an adaptive response to counterbalance the oxidative stress/tissue damage present in the inflammation site that contribute to metabolic dysbalance and subsequent cachexia.

Cellular metabolism and immunity

Cellular bioenergy and metabolism play important roles in regulating acute inflammation and immunity. Early innate and adaptive immune responses require a high-energy status supported by glycolysis-dependent production of ATP and activation of NADPH oxidase enzymes to kill microorganisms by ROS species (Kuwabara et al., 2015). In contrast, the later stage of acute inflammation is a low energy response (Singer, 2008), which supports healing to eventually restores homeostasis. Recent studies report in TLR4 stimulated THP1 promonocytes a switch from increased glycolysis to increased FA oxidation as early inflammation converts to late inflammation (Liu et al., 2012). Exacerbated lipolysis leads to lipid-induced insulin resistance, mitochondria overload and a switch to increased FA oxidation (Koves et al., 2008). Importantly, passage from exacerbated glycolysis to FA oxidation in life-threatening inflammation has

been recently reported to repress innate and adaptive immunity (Hotchkiss et al., 2009) in human spleen and lung from deceased sepsis patients (Boomer et al., 2011). However, the signaling pathways that initiate or terminate accelerated fat and muscle catabolism associated with a resolving/protective or exacerbated/harmful immune response remain unknown.

Pro-inflammatory mediators and cachexia

Lipid metabolism is altered during an acute inflammatory response. LPS-induced inflammation causes acutely increased serum TAG levels (2-24h after administration) and cholesterol by mechanisms involving increased lypolisis and suppression of FA oxidation (Feingold et al., 1992). Besides, systemic inflammation has been shown to result in increased protein catabolism and altered protein synthesis (von Allmen et al., 1992). On the other hand, several proinflammatory cytokines (IL-1-β, TNF-α and IL-6) have major roles in appetite regulation and energy homeostasis. Importantly, IL-6 and TNF-α are potent inducers of lipolysis.
In the case of cancer-associated cachexia, pro-inflammatory cytokines such as TNF-α had been proposed as an initiating trigger. However levels of circulating TNF-α are not consistently elevated in individuals with cachexia (Smemo et al., 2014). Nevertheless, TNF-α has a crucial importance in the pathogenesis of IBD, as demonstrated by the impressive results of clinical trials with biological agents targeting TNF-α (Steenholdt, 2016). However, TNF-α was not the most elevated cytokine in the serum of DSS-induced-colitis animals, neither in the ones that developed wasting. TNF-α mRNA levels were high in all DSS treated groups, including in colitis remitting mice, in both colon and spleen. Similarly, myeloid subsets analyzed by FACS showed increased TNF-α intracellular levels in both acute disease phase and colitis remission. Noteworthy, TNF-α is a strong inducer of lipolysis. This suggests that animals that are able to resolve colitis can probably control the lipolysis process, using the resulting metabolites for high energy-demand tissue repair and resolution of inflammation. In contrast cachectic animals with an exacerbated lipolysis might get to a non-return point.

Fatty acid metabolism and cachexia

Cachexia was formerly accepted as a chronic inflammatory state characterized by changes in lipid metabolism, total oxidation of FAs, hyperlipidaemia, hypertriglyceridaemia and hypercholesterolaemia, thought to be cause, partly, by increased inflammatory mediators in WAT (mainly TNF-α) with the potential of altering homeostasis of the tissue. A chronic inflammatory environment, such the present in advance cancer disease, may lead to a systemic hypercatabolic status, with changes in lipid and protein metabolism and the resulting severe loss in total fat mass and muscle (Batista et al., 2012).

Noteworthy, recently Fukawa and colleagues revealed that alteration in FA metabolism itself, as the first trigger of cachexia. Importantly, RORC has a role in regulation of lipid metabolism and the adipogenesis process. Of relevance, as a NR acts as a ligand-dependent TF, thus circulating FAs, as a result of

inflammation-mediated alteration of lipid metabolism in several tissues, might be sensed by RORC molecules in myeloid hematopoietic precursors leading to the generation of immature PMN-MDCSs not only in cancer but in chronic inflammatory conditions such as IBD. This might lead to a failure in protective innate immune responses and consequently, susceptibility to secondary infections and reduced fitness.

MDSCs and cachexia

A chronic inflammatory state of the host, such as in advance cancer disease, may lead to a systemic catabolic state, with increased lipolysis and release of free lipid metabolites, resulting in severe loss in total fat mass and consequently wasting. Fukawa et al. have proposed that inflammatory mediators (mainly TNF-α and IL-6) can directly alter mitochondrial oxidative metabolism in skeletal muscle, leading to energy inefficiency, ROS-induced-cellular stress such DNA damage and ultimately impair muscle growth leading to wasting Furthermore, these free FAs have the potential of inducing MDSCs (Yan et al., 2013), which can suppress protective innate and adaptive immunity, leading to increased susceptibility to secondary infections and death.

Ci-sCG and cancer cachexia

Confirming our results in DSS-associated-cachexia, we observed increased expression of the PAR-bZIP-CCGs genes and of the Per 1-3, in the context of cancer cachexia (AD). No differences were observed in the core components Clock and Bmal1. However, in this case an increased expression of the aforementioned TFs was coincident with higher expression of TNF-α and IL-6, both cytokines that are strong inducers of lipolysis.

Mounting evidence suggests that the circadian clock may function as a tumor suppressor at the systemic, cellular, and molecular levels, through its involvement in cell proliferation, apoptosis, cell cycle control, and DNA damage response. In addition to regulating other circadian genes, NPAS2:BMAL1 heterodimers have been shown to affect expression of the proto-oncogene c-MYC , implying a potential involvement of *NPAS2* in tumorigenesis (Hoffman et al., 2008). Hoffman et al. showed that depletion of NPAS2 had an impact on cell cycle progression and DNA repair capacity, supporting the epidemiologic association between NPAS2 polymorphisms and cancer risk (Hoffman et al., 2008). Furthermore, it has been shown that *NPAS2* silencing decreases cell viability, results in aberrant cell cycle response to DNA damage, and decreases DNA damage repair capacity, without altering the apoptotic response. Thus, in the context of advance cancer disease and in cachexia, as previously reported (Strauss et al., 2015a), Rorc1 and Npas2 expression in TAMs may promote their polarization to M2, as well as potentiate cell survival by increasing DNA damage repair capacity, respectively, to favor the tumor microenvironment. This may be as well supported by their higher expression in Irf8. Besides, we have reported an increase of NPAS2 in the spleen of DSS cachectic mice, which correlated with an accumulation of $RORC^{hi}$ PMN-like-MDSCs at the

expense of Ly6C^{hi} monocytes. This PMN-MDSC-like cell accumulation might be due to an increase production from the hematopoietic compartment due to the stress-induced environment, or rather due to a defect in their clearance. Although we did not address this question, it is known that RORC promotes survival of MDSCS , protecting them of apoptosis(Strauss et al., 2015b) . Further, apoptosis of neutrophils by macrophages is critical for restoring tissue homeostasis in the inflammatory site. Besides, we have reported NPAS2 expression in DSS-associated cachexia as well as in TAMs from advance cancer disease. Thus, it is plausible that NPAS2 and RORC expression in PMN-like-MDSCs may contribute to the survival of this population, perpetuating an immunosuppressive status in cachexia, that might contribute to poor survival due to the occurrence of secondary infections.

Recent work has shown that selective depletion of wound macrophages results in increased numbers of neutrophils and elevated inflammatory cytokine production at the site of injury, suggesting that this macrophage population plays a role in maintaining localized neutrophil homeostasis in the wound setting (Gordy et al., 2011). Although we did not observe a reduction in F4/80 macrophage numbers in DSS colitis, we reported a decrease in monocyte populations as well as a reduction in Irf8 expression in cachexia, which may limit the source of tissue macrophages in the inflamed tissue, leading to impaired control of neutrophil phagocytosis and tissue repair in order to resolve inflammation.

Fat browning and muscle wasting in cachexia

Cachexia is an energy-wasting syndrome, in which energy intake is decreased and/or energy expenditure is increased. It occurs in patients with chronic inflammatory diseases and being present in 80% of individuals with advance cancer stage, it is one of the primary causes of morbidity and mortality associated with cancer (Tisdale, 2002) . As previously mentioned, cancer-associated cachexia (CAC) is characterized by BW loss, atrophy of white adipose tissue (WAT) and skeletal muscle. Petruzzelli et al. showed that in the initial stages of CAC there is a switch from WAT o brown fat (phenomenon termed WAT browning), that precedes to muscle atrophy and that is associated to increased expression of uncoupling protein 1 (UPC1), which uncouples mitochondrial respiration toward thermogenesis instead of ATP synthesis, leading to increased lipid mobilization and energy expenditure in cachectic mice. Chronic inflammation (characterized by leukocytosis with increased neutrophil/lymphocyte ratio) and IL-6 increase UCP1 expression in WAT.

Loss of myofibrillar proteins in muscle cells is of key relevance in cancer cachexia as it results in muscle weakness and fatigue. Abnormalities in protein (synthesis and degradation) and amino acid metabolism (transport and branched-chain amino acid oxidation) have been observed in cachectic muscle. Furthermore, an increase in apoptosis and an impaired capacity for regeneration contributes to muscle wasting. All these alteration clearly contribute to a negative nitrogen balance (Argiles et al., 2014).

The pathway that seems to be most involved in the wasting process is protein degradation, which is mediated by the activation of the ubiquitin-dependent proteasome pathway (Argiles and Lopez-Soriano, 1996).
It is known that pro-inflammatory cytokines, such as TNF-α result in the stabilization and activation of PGC-1α (through p38 MAPK activation). Furthermore, overexpression of PGC-1α leads to increased expression of genes linked to mitochondrial uncoupling and energy expenditure (Argiles et al., 2014; Miura et al., 2006).

More recently, Fukawa et al. showed *in vitro* that cachectic cells secreted many inflammatory factors that rapidly led to high levels of FA oxidation and to the activation of a p38 stress-response signature in skeletal muscles, before manifestation of cachectic muscle atrophy occurred. Pharmacological blockade of FA oxidation rescued human myotubes and improved muscle mass and BW in CAC models *in vivo (Fukawa et al., 2016)*.

Cachexia and obesity

Both excess and deficiency of adipose tissue result in severe metabolic disturbances. Apart for having a role in myelopoiesis, the C/EBP family of TFs (specifically C/EBPβ and C/EBPδ) rise early in adipocyte differentiation and activate the expression of the NR Peroxisome proliferator-activated receptor-γ (PPARγ), the ‘master regulator' of adipogenesis, which in turn switches on transcription of C/EBPα (Kumpf et al., 2012).
Importantly, Wolfrum group identified RORC as a negative regulator of adipocyte differentiation, affecting the expression of key regulators of adipogenesis, such as PPARγ and the the C/EBP family members. RORC-/- had decreased adipocyte sizes and were protected from hyperglycemia and insulin resistance in the state of obesity.
It has been described that hyperthopic adypocytes are insulin resistant and change their secretory profile towards pro-inflammatory adipocytokines, resulting in lypolytic release of fatty acids, leading to cytotoxic fatty acid accumulation in extra-adipose tissue (Meissburger et al., 2011).
Increased RORC expression has been reported in visceral adipose tissue of obese patients and it is reported an upregulation of its expression in late stages of adipocyte differentiation, suggesting that RORc may influence the progression of obesity associated insulin resistance in mice and humans (Meissburger et al., 2011).
We reported RORC as cachexia promoting factor in the context of acute colitis, as RORc-/- seems to be resistant to DSS-associated-cachexia. Although we did not asses the study of adipose tissue and skeletal muscle, we could expect muscle and fat wasting, due to the excessive BW loss observed in these mice which may be a result of increased lypolisis and free FA levels in the circulation, leading to downstream responses like glycolysis inhibition and activation of the p38 MAPK pathway, resulting in muscle wasting.

Although the primary cause might be different, obesity and cachexia have shared features such as chronic inflammation, with the consequent systemic level of circulating inflammatory factors, as well as increased hyperlipidemia and its associated toxicity for adjacent tissues due to free FAs in circulation. Interestingly, RORC-/-has been showed to be protected from diet-induced-obesity(Meissburger et al., 2011), and we have here reported for the first time that it is as well protected form DSS-associated cachexia.

Similarity of sepsis and cachexia

Interestingly, similar to sepsis, in DSS cachexia we observe a complicated scenario with the overlapping coexistence of inflammatory and immunosuppressive processes. Sepsis is a complex pathology that arises from dysregulated host inflammatory responses to systemic bacterial infection .In line with the dynamic nature of sepsis, two phases have been recognized in this disease: an early phase characterized by leukocyte activation, cytokine storm, and a systemic inflammatory response; and a later phase characterized by immunosuppression, leukocyte deactivation, increased risks of secondary infection, and high mortality (Hotchkiss et al., 2009; Shalova et al., 2015).

Recent evidence has emphasized how immunosuppression might contribute to increasing mortality risk in most sepsis patients, contrasting the common view that overt inflammation drives mortality in sepsis (Boomer et al., 2011; Hotchkiss et al., 2009; Shalova et al., 2015). Nonetheless, the cellular and molecular mechanisms that regulate different aspects of human sepsis pathogenesis still remain poorly understood.

We have reported in DSS cachexia increased pro-inflammatory cytokine levels in the inflammatory site (TNF-α, IL-1-b, IL-17A, IL-6), as well as in spleen and serum (TNF-α, IL-6, IL-27), but at the same time high levels of anti-inflammatory cytokines like IL-10 in colon or IL-22 in serum. Besides, we have observed overexpression of growth factors important for myelopoiesis (G-CSF, GM-CSF and M-CSF), as well as regulators of the process (increased C-EBP/b, reduced SOCS3, bcl3 and IRF8) but presence of immature-like myeloid subsets of granulocyte nature in the periphery, characterized by high RORC1 expression (and accumulation of $RORC^{hi}$ GMP progenitors in BM; accompanied by a reduction in $LY6C^{hi}$ splenic and colonic populations (which express RORC as well, which is know to polarize M1 to M2 macrophages) leading to a failure in colitis resolution and death.

Shalova et al. concluded form their experiments in human sepsis that HIF-1α (via IRAKM upregulation) functionally re-programs monocytes to an immunosuppressive phenotype characterized by a defective pro-inflammatory response to endotoxin and skewing T cells to Tregs (Shalova et al., 2015).

In contrast to these observations, in our DSS cachexia model we had no Treg induction in spleen nor colon, despite of colon/spleen elevated mRNA levels of IL-10 and IL-22 in serum. However, we observed high IL-6 levels in spleen and serum and decreased TGF-beta gene expression, supporting our observation, as decreased IL-6 and increased TGF-beta are key determinants for Treg polarization. Furthermore, Il-10 was not expressed in splenic or colonic myeloid populations (data not shown). Nevertheless, DSS model of

acute colitis it is known for not presenting a T cell induction, so this might be the reason why we observed low T cell numbers I general (both in colon and spleen, as well as low CLP precursors in the BM (data not shown), and thus no Treg induction.

'Emergency' myelopoiesis in DSS-associated cachexia and advance cancer disease

Hematopoietic stem cells (HSCs) can self-renew and also give rise to the entire repertoire of hematopoietic cells. Immunologic stress, such as infection and cancer, modifies the magnitude and composition of the hematopoietic output, a feature of immune regulation defined as 'emergency' hematopoiesis, in order to guarantee proper supply of immune cells to increased demand (Ueha et al., 2011). Thus, during inflammatory stresses, the hematopoietic system can quickly adapt to demand by increasing output of innate immune cells many-fold, often at the expense of lymphopoiesis and erythropoiesis. Powrie and colleagues showed that intestinal inflammation had a substantial impact on the most primordial steps of hematopoiesis, with HSC proliferation (LSKs) was increased in the BM of colitic mice, and striking LT-HSC accumulation. During colitis, the cell composition inside the myeloid progenitor population in the BM was dramatically, with a skew toward GMP differentiation, at the expense of both MEP and CLP differentiation (Griseri et al., 2012), supporting our observations in DSS-induced colitis and cachexia. We report in DSS-colitis an increase of GMPs at expense of MEPs in the BM, and this is correlated with disease severity and cachexia/death. Of note, Griseri et al. showed that transfer of GMPs exacerbated colitis (Griseri et al., 2012).

Coordinated actions of cytokine signaling and TFs promote one cell fate while inhibiting the alternative cell fate (Friedman, 2007). Differentiation of GMPs into granulocytes or monocytes is a mutually exclusive cell fate decision (Akashi et al., 2000b). G-CSF induced granulopoiesis is mediated through the transcription factors c-EBPβ (Akagi et al., 2008) and STAT3 (Zhang et al., 2010), whereas M-CSF supports monocyte differentiation through the TFs PU.1 and IRF8 (Friedman, 2007). Irf8 in conjunction with PU.1 activates gene transcription that promotes monocytic differentiation while inhibiting gene transcription that promotes granulocytic differentiation. Loss of Irf8 transcriptional activity results in accumulation of GMP, defective monocytic development and enhanced granulocytic differentiation (Kinder et al., 2010). We reported decreased Irf8 levels in the BM of cachectic mice, which supported the increased percentage in neutrophils at the expense of inflammatory monocytes populations. Besides, IRf8 levels increased from d9 of acute colitis to d14 of disease remission, which was accompanied by an increased in monocytic spleen and colonic population.

The low basal migration of HSCs from the BM to the periphery is a well-established phenomenon, but its functional relevance is unknown (Kondo et al., 2003). However, peripheral HSCs may contribute to emergency extramedullary hematopoiesis within infected/inflamed tissues (Massberg et al., 2007). Pathological conditions are generally associated with a profound accumulation of mature myeloid cells

both in the BM and extramedullar tissues (Pu et al., 2016). We did not investigated the presence of HSCs or HSPCs and their proliferation capacity in the periphery, but the reported BM accumulation of immature PMN progenitors and of neutrophils in colon and spleen, both characterized by high RORC expression, may be an indication of extra medullar myelopoiesis from HSCs or HSPCs in these organs, rather than their egression from BM and migration into these tissues, as it has been reported during systemic inflammation (Zhao and Baltimore, 2015). Because GMPs and mobilized HSCs have high proliferative indexes, their accumulation in the inflamed spleen constitutes a reservoir of HSPCs capable of engaging in extramedullary hematopoiesis, which may contribute substantially to the increase of peripheral neutrophils and monocytes that promotes intestinal inflammation (Griseri et al., 2012). Interestingly, Swirski and colleagues described that high numbers of undifferentiated monocytes reside in the spleen, outnumbering their equivalent in the blood, and these splenic monocytes rapidly migrate to tissues upon inflammation giving more weight to our reported reduction in the monocyte subset in the spleen/colon of cachectic mice, in comparison to non cachectic, during acute colitis (Swirski et al., 2009). In line with this, Powrie et al. reported the spleen to constitute a reservoir of HSCs and GMPs that might contribute to the increase in splenic myeloid cells and their likely migration to the colon, as a result of their expression of a4b7 integrin (Griseri et al., 2012). This is as well supported by the observation form Massberg et al. who detected a scarce population of recirculating HSPCs, which constantly surveys non-lymphoid tissues and can trigger local urgent hematopoiesis upon TLR stimulation (Massberg et al., 2007).

Of note, TLR signaling drives Myd88-dependent myeloid differentiation of primitive hematopoietic cells (Nagai et al., 2006). Interestingly, not only hematopoietic cells nor stromal cells, but endothelial cells (ECs) from multiple tissues, including BM, express high levels of Tlr4 and Myd88 and are the primary source of granulocyte colony-stimulating factor (G-CSF) after LPS challenge or infection with E.coli. EC-intrinsic MYD88 signaling and subsequent G-CSF production by ECs is required for myeloid progenitor lineage skewing GMPs, increased colony-forming unit (CFU) granulocyte activity in BM, and accelerated BM neutrophil generation after LPS stimulation. Thus, ECs catalyze the detection of systemic infection into demand-adapted granulopoiesis (Boettcher et al., 2014).

The observed increase of HSCP in the BM (and most likely spleen) could be potentially beneficial for the host during microbial infection, but detrimental in chronic inflammation or cachexia, as HSCPs have reported to be highly proliferative, providing rapid and local source of myeloid cells that may sustain inflammation and perpetuate disease (Griseri et al., 2012). Enhanced activity of self-renewing HSC might be crucial for the chronicity of increased myelopoiesis during colitis cachexia, as it sustains the supply of short-lived medullary and peripheral GMPs (Passegue et al., 2005).

Ueda and coworkers reported that HSCs express receptors for IFNs, IL-1, and IL-6, being therefore poised to respond promptly to various inflammatory stimuli (Ueda et al., 2009). IFN-γ is a well-known colitogenic cytokine that is increased in the colon and serum of colitic mice and its secretion by pathogenic Th1 and Th17 cells and ILCs is promoted by IL-23, suggesting that IFN-γ may exert its pathogenic role in colitis by boosting the hematopoietic activity at the level of the primordial HSCs, and sustain the increased

production of GMPs (Griseri et al., 2012). Accordingly, we observed in all DSS treated groups increased IFN-γ in serum, although the differences were not significant between cachectic and non cachectic, and mice in remission and Powrie et al. described an amelioration of colitis and a significant reduction of HSCS in BM and spleen of colitic mice, after IFN- γ blockade (Griseri et al., 2012).
On the other hand, Libregts et al. showed that on IFN-γ stimulation, IRF-1 binds to the promoter of PU.1, leading to inhibition of erythropoiesis, (Libregts et al., 2011) supporting our data on the reduction in MEP populations in DSS animals during the whole disease course and cachexia.

CSFs and 'emergency' myelopoiesis response

Colony-stimulating factors (CSFs) are major orchestrators of hematopoietic development. Among these, granulocyte CSF (G-CSF) and granulocyte-macrophage (GM-CSF) drive 'emergency' myelopoiesis by securing supply of neutrophils and macrophages from bone marrow (BM) and hematopoietic stem cell niches (HSCs)(Metcalf, 2008; Ueha et al., 2011). Further, the macrophage CSF (M-CSF) promotes macrophage differentiation from medullar precursors and differentiation of tissue macrophages involved in tissue homeostasis (Hume and MacDonald, 2012) and tumor progression (Qian and Pollard, 2010). We reported an increase of all three growth factors in cachectic mice, although the most striking increase was observed in colonic and splenic expression G-CSF. Monocytes can give rise to either DCs or macrophages, depending on the surrounding inflammatory environment, GM-CSF and IL-4 or IL-6 and M-CSF respectively. This differentiation is initiated when monocytes cross the endothelium. In turn, IL-6 up-regulates the expression of functional M-CSF receptors on monocytes, allowing them to respond in an autocrine way to M-CSF. Thus, the interplay between IL-6 and M-CSF switches monocyte differentiation to macrophages rather than DCs (Chomarat et al., 2000), which would explain our observation in the spleen of DSS treated mice, were no differences in the percentage of cDCs populations, while, $F4/80^{+}$ macrophages increased significantly from d7 of DSS treatment onwards.
Interestingly, GM-CSF is thought to drive the HSCs away from CLP development in favor of CMPs and later promote both proliferation and survival of GMPs (Kimura et al., 2009; Kondo et al., 2003), accordingy, we observed an increase of GM-CSF mRNA levels in cachectic mice. Besides, GM-CSF-R is expressed by CMPs and GMPs, but absent from MEPs and CLPs (Griseri et al., 2012).
Alternatively, G-CSF guides the differentiation from HSCs to granulocyte precursors through the activation of G-CSFR on HSCs (Zhang et al., 2010). In homeostatic conditions, G-CSF–G-CSFR signals maintain mature neutrophil numbers in circulation by regulating the proliferation and differentiation of myeloid progenitors as well as survival of postmitotic bone marrow neutrophils. Schwarzenberger and colleagues showed that IL-17-mediated granulopoiesis requires G-CSF release and the presence or induction of the transmembrane form of stem cell factor (SCF, c-kit ligand) for optimal granulopoiesis (Schwarzenberger et al., 2000). We reported as well high IL-17A and G-CSF expression in the spleen of DSS treated animals, and IL-17 has been reported to stimulate hematopoietic activity in the BM via stimulation of G-CSF

secretion by stromal cells, and in the spleen via G-CSF dependent and independent mechanisms (Schwarzenberger et al., 2000).

NAMPT and 'emergency' myelopoiesis

NAMPT is essential for the G-CSF–induced myeloid differentiation via a NAD^+-SIRT1-dependent pathway in both healthy individuals and in individuals with severe congenital neutropenia. Intracellular and extracellular administration of NAMPT, as well as administration of vitamin B3 (nicotinamide, NAMPT substrate) leads to granulocytic differentiation of $CD34^+$ hematopoietic progenitors. Besides, the treatment of healthy individuals the NAMPT substrate. The molecular events triggered by NAMPT include NAD^+-dependent SIRT1 activation, subsequent induction of C-EBP-a and C-EBP-b, and finally up-regulation of G-CSF (which in turn increases NAMPT levels) and G-CSF-R receptor expression (Skokowa et al., 2009). Noteworthy, it has been described that NAMPT is up-regulated during neutrophil activation and acts as an anti-apoptotic factor for neutrophils at least in the context of clinical and experimental sepsis (Skokowa et al., 2009). Noteworthy, we report increase levels of *nampt* and *g-csf* in cachexia, as well as accumulation of splenic and colonic $RORC^{hi}$ neutrophils as well as $RORC^{hi}$ BM GMPs. Interestingly; we also observed increased Nampt levels in in sorted splenic neutrophils from cachectic mice. Furthermore, TNF-a and IL-6 are known to induce NAMPT expression (Skokowa et al., 2009). In accordance, we observed high levels of these pro-inflammatory cytokines in the serum of cachectic mice.

Importantly, RORC -/- mice exhibit decreased *Nampt* and *G-csf* levels compared to their WT littermates. Further, they show decreased neutrophil numbers in colon and spleen and GMPs do not increase in the acute phase of the disease, as in the WT animals, and they are protected form DSS-associated cachexia.

On the other hand, DNA damage in stromal/epithelial cells leads to G-CSF release, triggering the activation of CXCL12–CXCR4 chemotactic pathway. In addition to stimulating neutrophil proliferation, G-CSF mobilizes neutrophils from the BM through proteolytic disruption of the chemokine receptor CXCR4 and its chemotactic ligand CXCL12 (Epstein, 2004). Accordingly, to the previous observations, we observed increased levels of Cxcl12 in cachectic spleen, and importantly, the levels in RORC -/- were significantly decreased.

Alternatively, IL-27, one of the interleukin (IL)-6/IL-12 family cytokines, plays an important role in emergency myelopoiesis. Among various types of hematopoietic cells in bone marrow, IL-27 promotes the expansion of LSK cells, especially LT-HSCs, and differentiation into myeloid progenitors in synergy with SCF, with an enhanced potential to differentiate into neutrophils (Furusawa et al., 2016). Of note, we reported a significative increase of IL-27 serum levels in DSS treated animals, although no differential expression of this cytokine was observed in DSS-induced-cachexia.

RORC and 'emergency' myelopoiesis

Strauss et al. reported that RORC1-/- tumor bearing mice supported effectively emergency hematopoiesis while displaying defective induction of MDSCs, leading to an inhibition of splenic MDSCs expansion, reduced tumor growth, and decreased number of metastasis. Instead, these mice showed an expansion of the granulocytic compartment and a contraction of the erythroid hematopoietic colonies. Furthermore, treatment of tumor bearing mice with RORC1 agonist (SR1078) increased metastatic burden as well as promoted the expansion of M and PMN-MDSCS. In contrast to our observation in DSS colitis cachexia, BM progenitors form RORc-/- failed to differentiate to macrophages, having an increased in granulocyte numbers. Furthermore, the authors showed that RORC1 signalling leads to terminal differentiation and M2-polarization of TAMs , hampering neutrophil accumulation in the tumor. Importantly, they described increased spleen and BM expression of potent inhibitors of emergency myelopoiesis, SOCS3 and Bcl3, as well as a decrease of the master regulator of this process, C/EBP-beta, supporting the involvement of RORC in stress-driven granulopoiesis, and its deleterious expression in the context of cancer, promoting tumor growth and metastasis. In our model of experimental acute colitis , RORC-/- mice exhibited less severe disease course and importantly, were protected from DSS-induced cachexia.

The observed expansion of peripheral granulocyte population in cachectic mice was at the expense of monocyte populations, which could be explained by the observed increased in BM GMPs in the BM, together with the reported increased levels of *G-csf* and decreased expression of *Irf 8* .

Interestingly, RORC deficient mice exhibit $Ly6C^{hi}$ monocyte recruitment to the inflamed colon, which persisted in disease remission and showed no Ly6G cell infiltration in the inflamed colon nor in the spleen during the course of DSS experimental colitis, in contrast to WT cachectic animals. This was as well supported by their significantly reduced expression of G-CSF and NAMPT in comparison to their WT counterparts. Finally, RORC mice did not showed Ci-sCG signature observed in WT cachectic mice and they were protected from cachexia. In contrast, TFs involved in epigenetic control to DNA damage and metabolic stress such as Npas2, Per3, Hlf were significantly down-regulated in spleen from RORC depleted animals in the acute phase of DSS colitis, and their levels remained decreased at d14 of disease resolution. Supporting this observation, Takeda et al. reported previously that RORC deficiency reduced the peak of expression of several clock genes in a tissue –selective manner without significantly affecting the phase of their rhytmic expression patterns. Furthermore, the transcriptional activation by RORs through ROREs was associated with changes in histone acetylation and chromatin accessibility (Takeda et al., 2012). It is known that the nutritional status affects the cellular redox balance, specifically, NAD/NADH ratio, leading to direct effects on the deacetylase SIRT1 and nampt oscillatory expression. Of note, sIRT1 can also deacetylate and regulate proteins involved in metabolism and cell proliferation. This might results in a differential binding capacity of the core clock heterodimers into the promoter of CCGs, which might explain partly, the reduced peak of clock gene expression in the RORC -/-.

Chapter 5.

CONCLUSIONS

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We report for the first time that clock gene expression patterns in splenic myeloid cells associate with the outcome of acute experimental colitis. Ci-sCG is selectively observed in inflammatory monocytes and neutrophils in resolution of DSS-induced-colitis, while Ci-sCG is lost in mice that develop DSS-associated cachexia, and fail to recover after DSS withdrawal. However, locomotor activity remains decreased in mice undergoing colitis remission. Intriguingly, pro-inflammatory cytokine levels in the inflamed tissues do not differ significantly between the experimental groups, and elevated levels persist during resolution of acute colitis. Importantly, high TNF-a dosages are not able to revert Ci-sCG, indicating that clock gene regulation in inflammatory diseases might implicate other mechanisms than pro-inflammatory cytokines. The characterization of the molecular clock in patients with IBD may identify patients at risk for a severe course of disease and cachexia.

RORC is a NR with a putative role in mediating 'emergency' mono-granulocytopoiesis, a systemic stress-induced immune response that is a key driver of chronic inflammatory diseases, including cancer and colitis. We report RORC expressing monocytes and neutrophils in two models of chronic inflammation: DSS colitis and advance cancer disease. We observe in both models important BW loss and up-regulation of clock gene expression. DSS-associated cachexia leads to an expansion of colonic and splenic $RORC^{hi}$ neutrophil populations at the expense of $Ly6C^{hi}$ inflammatory monocytes (which also express RORC), as well as an accumulation of $RORC^{hi}$ GMPs in the BM. In the chronic cancer inflammation model, this population enriched in neutrophils and poor in inflammatory monocytes, express high levels of RORC and present a MDSC-like-phenotype. Furthermore, we observe a down-regulation of *Irf8* in cachectic BM, which has been reported to be an inhibitor of MDSCs development, by driving the fate of GMP and neutrophils to effector monocytes. In fact, we observe that $RORC^{hi}$ neutrophils and GMPs and neutrophils do not accumulate in remission of acute colitis. In contrast, we observe an increase of Ly6Chi monocytes and splenomegaly, both being sign of an ongoing pro-inflammatory reaction. Besides, IL-23 is elevated in colitis remission, cytokine that promotes monocyte recruitment.

Previous studies in the context of intestinal inflammation have shown that GMP accumulation promotes disease severity and progression. We report for the first time an enrichment of $RORC^{hi}$ GMPs in the BM from DSS-cachectic mice and increased presence of $RORC^{hi}$ PMN-MDSC-like cells in the spleen and colon of these animals.

Finally, we report a novel implication of RORC driven DSS-triggered 'emergency 'granulopoiesis contributing to cachexia development. Noteworthy, RORC deficient mice present less severe clinical and histological colitis score, compared to their WT counterparts, and importantly, do not develop DSS-associated cachexia. Furthermore, in contrast to RORC deficient mice, cachectic WT up-regulate G-CSF and CXCL12, both drivers of emergency granulopoiesis. Moreover, RORC -/-mice show a failure in inflammation-induced 'emergency' myelopoiesis, with no accumulation of neutrophils nor GMPs.

Interestingly, amplitude of clock gene expression in RORC-/- is reduced.

Thus, these results suggest that circadian adaptation of immune and energy metabolism to inflammatory stress might have a crucial role in resolution of inflammation and survival. How these complex networks operate remains unknown and should be further investigated.

Chapter 6.

EXPERIMENTAL PROCEDURES

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6.1 Ethics statement

Animal experiments were performed according to Swiss animal welfare laws and were approved by the veterinary authorities of Zürich, Switzerland (Kanton Zürich Gesundheitsdirektion Veterinäramt, approval no. 76/2014).

6.2 Animals

Eight to ten-week-old female C57BL/6J-Crl mice (Charles River, Germany) or RORC-/- (Jackson) were used for the experiments and housed in individual ventilated cages on 12:12 light:dark cycle at 22°C, with *ad libitum* access to food and water. Before every experiment we allowed mice at least 7 days of acclimatization to a LD cycle (light on at 0700 Zeitgeber (ZT) 0; light off at 1900 ZT12).

6.3 Induction of experimental colitis and analysis of clinical parameters of disease.

DSS-induced Colitis Model (*)

We chose the DSS model of colitis as we wished to examine the role of Clock genes and RORC expression in the early stages of colitis, when the innate immune response may be more relevant. This model does not depend on the adaptive immune system, given that immunodeficient mice are susceptible to DSS injury (Dieleman et al., 1994). Antibiotics can ameliorate the severity of DSS-induced colitis (Hans et al., 2000; Rath et al., 2001). Thus these historical data support the use of the DSS model to query the role of the innate immune response in the development of acute colitis.

Eight to ten-week-old female mice received 2.5% DSS (MP biomedicals, Illkirch, France) in their drinking water for 7 days (Menzel et al., 2006). After DSS withdrawal (1 cycle of 7 days) animals received normal drinking water for additional 7 days. Animals were monitored daily and colitis severity was assessed for BW, stool consistency and rectal bleeding. Mice were euthanized using CO_2 at day (d) 7, 9 and 14 starting at ZT 0, alternating the control and experimental group.

Assessment of disease activity. Body weight was assessed at baseline and every day for the duration of the experiment. Weight change was calculated as percent change in weight compared with baseline.

*To address the time point of maximal injury, DSS treatment was continued for a total of 7 days followed by 7 days without DSS, i.e., recovery. The degree of epithelial damage as well as clinical colitis score was most severe between days 8 and 11 and was minimal by day 14.

6.4. Colon length, acute colitis score and histological analysis

A reduction in the colon length reduction was used as an indirect parameter of colonic inflammation, as described by (Axelsson et al., 1998) . For histological analysis, the colon was removed, mechanically cleaned, and measured to 0.1 cm precision. The distal cm of the colon was then cut longitudinally, laid on a filter paper and fixed in 10% formalin overnight. 5 μm sections of the paraffin-embedded material were cut serially and stained with haematoxylin-eosin.

Histological sections were scored in the following way: Epithelium (E): 0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas. Infiltration (I): 0, no infiltrate; 1, infiltrate around crypt basis; 2, infiltrate reaching to *L. muscularis mucosae*; 3, extensive infiltration reaching the *L. muscularis mucosae* and thickening of the mucosa with abundant oedema; 4, infiltration of the *L. submucosa.* The total histological score represents the sum of the epithelium and infiltration score (total score= E+I) (Hausmann et al., 2007). Histological scoring of the individual mice was performed in a blinded fashion.

6.5. Locomotor and temperature monitoring

Six-to–eigth-week-old female mice were housed in individual cages in a temperature-controlled sound-proof light-tight room. Food and water were available ad libitum. We allowed mice 7 days of acclimatization to a LD cycle (light on at 0700 ZT=0; light off at 1900 ZT=12). Mice were operated under deep isofluorane anaesthesia two weeks prior to induction of colitis with DSS.

Locomotor activity and temperature was assessed by intraperitoneal (i.p) implantation of telemeters (TA-F10, DSI) under sterile hood and by the use of an optic microscope connected to a camera. The hair from the ventral side of the mice was removed previously to avoid contamination. Briefly, a 1 cm skin incision was performed with autoclaved scissors, followed by a cut in the peritoneum layer. Afterwards 1 mL of pre-warmed sterile saline solution was injected in the peritoneum with a sterile syringe, followed by the insertion of the DSI transmitter. Finally, peritoneum layer was surgical closed with absorbable sutures and skin layer was closed with non-absorbable sutures. This system allowed us to collect high-resolution continuous activity and temperature data in conscious free-moving animals in order to evaluate SBS. Telemetry recordings were based on mean average activity of 1 h intervals by using the Dataquest A.R.T. software.

6.6. Blood glucose monitoring during acute colitis course.

Blood glucose was determined in DSS treated mice at d7, d9 (cachectic and non cachectic) and d14, as well as in untreated animals between ZT 0-2. Blood samples were collected without anaesthesia from the distal tail. A ≤1-mm portion was amputated with a lancet, and blood (0.3 μL) was collected using capillary test strips (Nova Max glucose). Blood glucose concentrations (mmol/L) were measured using the Nova Max Link Blood Glucose Meter Kit (Nova biomedical) blood glucose monitoring system.

6.7. TNF-α injection experiment.

Recombinant murine TNF-α (2 ng or 60 ng diluted in PBS; Preprotech; Cat. N° 315-01A) or PBS as a control was administered i.p in female naïve mice. Animals were injected at two different time points of the day: ZT0, onset resting period or ZT12, onset of activity period. BW loss and welfare of the mice was monitored for 12h, and sacrificed at the end of the period, unless it was required before. Spleens were harvested and frozen in Peqgold (Peqlab, Germany) for subsequent RNA extraction.

6.8. Tumor Model Fibrosarcoma (Milano/Zürich)

Transplantable murine fibrosarcoma (MN/MCA1) cells (10^5) were inoculated intramuscularly in the left hind limb of female WT (C57BL/6 mice, Charles River, Italy). Tumour growth was monitored three times a week with a calliper, starting from day 14. After tumour cell injection (day 0), tumour volume (cm3) was measured starting from day 14 until day 28. MN/MCA1 bearing mice were classified into early- (ED) and late-stage (AD) tumour bearers.

6.9. Cell isolation

i- Colon lamina propria

Isolation of colonic LP cells was performed by following a method established previously with slight modifications (Spallinger M., USZ). The colon was dissected from the cecum until the rectum (anus). Extra-intestinal fat tissue was carefully removed and colons were then flushed of their luminal content with cold PBS. N=6 colon/group were pooled. The more distal cm colon tissue was preserved in Peqgold (Peqlab) at -80°C for future RNA extraction. The remaining colon tissue (proximal) was opened longitudinally and cut in small pieces (1-2 mm^3) in ice cold PBS. This was transferred to a 50 mL falcon tube containing 15 mL of wash buffer with EDTA solution **(Table 4)**. The tube was secured with parafilm and shacked at 200 rpm, 37°C, 30 min. Afterwards, the tube was shacked vigorously and poured through a 70 μM cell strainer on ice (to collect colon epithelial cells a posteriori). The colon tissue was poured back in the same tube with 20 ml ice cold HBSS, shacked 10x vigorously, followed by 1 minute of vortex. This step was repeated 3 times (saving each time the flow through for the epithelial cells). After, the tissue was transferred into a 50 mL Falcon tube containing 3 ml of the Digestion mix (**Table 4**), shacked vigorously and vortex for 1 minute. Next, it was stirred at 300 rpm for 15' min (37°C). Afterwards, it was shacked vigorously,vortex and filtered (70μm). The cell suspension was kept on ice with 5 mL of FBS (and inverted several times). The tissue was poured back again to the old tube with 3 mL of new digestion solution (this step was repeated 3-4 times). Eventually the tube containing the flow through was filled up with cold 0.5% BSA/PBS to 50 mL and centrifuged for 10 min at 1600 rpm (4°C). After, the supernatant was discarded and the pellet was resuspended in 10 mL of 10% IMDM and vortex. The cell suspension was then filtered

(70 µM) and washed with ca. 25ml 0.5% BSA/PBS. Finally the cell suspension was filtered (40µm) and washed with ca. 25ml 0.5% BSA/PBS *(optional if clumpy)*. *LP* cell suspension was enriched for mononuclear cells on a Percoll (Bioswisstech) density gradient (**Table 3**). After washing, the cell suspension was resuspended on 40% percoll solution and carefully loaded onto a 80% percoll gradient. The 2-layer solution was centrifuged for 30 min (RT) at 1700 rpm without break. The resulting cell suspension was washed and used for further procedures.

ii: Spleen

For cell isolation spleen was mechanically disrupted with the use of a syringe plunger and a 70 um cell strainer. Red blood cell (RBC) lysis was performed using 1mL of RBC lysis buffer and 8 min of incubation on ice, followed by a washing step with complete media and a second step with ice cold PBS. Unless stated otherwise, all centrifugation steps were carried out for 5 minutes at 500g/5°C. The cell suspension was filtered through a 40 um cell strainer and used for further procedures.

iii. Bone marrow

BM cells were flushed from femurs and tibias with complete medium (DMEM, 10% fetal bovine serum, 2 mM glutamine, 100 g/ml penicillin and streptomycin) and washed three times with complete medium. BM cells were RBC lysed (1mL, 8 min on ice), followed by a washing step with complete media and an additional wash with cold PBS. The cell suspensions were then filtered (40 µm) and used for further procedures.

iiii. Tumor-bearing mice organs

Mouse tumours were cut into small pieces, disaggregated with collagenase (0.5mg/mL) and filtered through cell strainers in order to obtain cell suspension, which was used for further procedures. For FACS analysis, cells (10^6) were stained with specific antibodies and with live/dead dye (Invitrogen, Life Technologies) and analysed by FACS instrument.

6.10. Flow cytometry analysis and Sorting of Phagocyte subsets.

Flow cytometry analysis (FACS)

Flow cytometric analysis was performed following standard methods, previously reviewed (McLaughlin et al., 2008; Perfetto et al., 2004). Flourochrome-conjugated monoclonal antibodies (mAbs) used were obtained either from eBioscience, BD, Biolegend or R&D biosystems (**Table 2**). The staining were performed in a 96-well plate with round bottom to optimize the reaction, and posteriorly transferred to FACS tubes, prior to the acquisition. For surface antigen staining, cells were incubated with the respective

mAbs fpr 20-30 min at 4°C. For intracellular staining, cells were incubated with BDCytofix/Cytoperm™ Fixation/Permeabilization Kit (Cat. No. 554714) following manufacturer instructions. For all experiments, dead cells were excluded from the analysis using Live/dead fixable staining reagent (Invitrogen) (Perfetto et al., 2010). Doublets were excluded by FSC-Area vs. FSC-Height and or SSC-Area vs SSC-Height gating. FACS analysis was carried out using LSR II Fortessa (BD) with FACS Diva software. Single stained compensation controls were prepared by using anti-rat/hamster Compensation beads (BD) or universal beads (eBiosciences) and the fluorophores used in the particular experiment. Cell sorting was performed by the FACS Core Facility (UZH) using FACSAria III (BD). Data analysis was performed using FlowJo 10.0.0.x (TreeStar).

6.11. Isolation of splenic inflammatory monocytes and neutrophils subsets for gene analysis.

i. $CD45^+$ $CD11b^+Ly6C^{hi}Ly6G^-$ monocytes
ii. $CD45^+$ $CD11b^+Ly6G^+Ly6C^{int}$ neutrophils

Mice were grouped into cachectic and non- cachectic animals, cachexia being defined as weight loss of ≥20% in the course of disease. Spleens from naïve and DSS treated mice (5-6/group) were harvested, weighted and processed mechanically into single-cell suspensions. Splenocytes were Red Blood Cell (RBC) lysed and counted. Negative magnetic bead selection prior to FACS sorting was used to enrich pooled splenocyte suspensions for the myeloid population by using anti-CD19-Miltenyi MicroBeads and an Automated Magnetic Activated Cell Sorter (auto MACS) (as described by the manufacturer). B cell (CD19) -depleted samples were filtered and resuspended in FACS buffer in polystyrene tubes. Spleen cell suspensions were FACS sorted for live (Far red Live/dead fixable staining 633-635 nm; Invitrogen) $CD11b^+Ly6C^{hi}Ly6G^-$ monocytes and/or $CD11b^+Ly6G^+Ly6C^{int}$ neutrophils.

6.12. Isolation of BM progenitors for gene analysis

i. Lin^- $CD127^-$ $Ska1^-$ $C\text{-}kit^-$ $CD34^+$ $CD16/32^-$ Common myeloid precursors (CMPs)
ii. Lin^- $CD127^-$ $Ska1^-$ $C\text{-}kit^+$ $CD34^+$ $CD16/32^+$ Granulocyte-monocyte precursors (GMPs)

Mice were grouped into cachectic and non- cachectic animals, cachexia being defined as weight loss of ≥20% in the course of disease. Femur and tibia from naïve and DSS treated mice were harvested, weighted and processed mechanically into single-cell suspensions. BM cells were Red Blood Cell (RBC) lysed (1mL, 8 min on ice), followed by washing step with complete media, followed by an additional wash with PBS. The cell suspensions were filtered (40 uM) and pooled (n=6 mice /group/day). A fraction of the pooled-cell suspension was counted by Tripan Blue staining using a Neubauer chamber, to estimate the number of live cells prior to the mAb staining. Immunomagnetic negative bead selection of mouse hematopoietic progenitor cells was used to enrich pooled BM suspensions (StemSep™ Mouse Hematopoietic Progenitor Cell Enrichment Kit; Cat n° 13056), as described by the manufacturer. Lineage

positive-depleted (80-90% purity) samples were filtered and resuspended in FACS buffer in polystyrene tubes. BM cell suspensions were gated on the living (Far red Live/dead fixable staining 633-635 nm; Invitrogen) LK population (Lin $^-$,CD127$^-$, Sca1$^-$, ckit $^+$,) and sorted for Lin $^-$,CD127$^-$, Sca1$^-$, c-kit $^+$,CD34 $^+$,CD32/16$^-$ (CMPs) or CD32/16$^+$ (GMPs).

6.13 RNA isolation and gene expression analysis

In preliminary gene expression studies the organs of interest were extracted at the onset of the resting phase (ZT = 0) and at the onset of the activity phase (ZT = 12). Colon, spleen or isolated BM cells were frozen in Peqgold (Peqlab, Germany) for subsequent RNA extraction.

Whole cell RNA from spleen and distal colon tissue or BM cells was extracted using NucleoSpin-RNA II kit (Macherey-Nagel). RNeasy mini kit (Qiagen) was used to get enough RNA out of FACS sorted subsets form spleen and BM. RNA purity was assessed with ND-1000 Nanodrop (Peqlab). RNA was reverse-transcribed using random hexamer primers (Thermo cientific) and M-MuLV reverse transcriptase (Life technologies). 1 μg of total RNA was amplified in Raptor detection system (as described by the manufacturer) using the SYBR PrecisionPLUS qPCR Mastermix (Primerdesign). The relative levels of each RNA sample were calculated by the 2-ΔCT method using Biogazelle, qbase plus software (Livak and Schmittgen, 2001); *HPRT1 and eEF1a1* were used as housekeeping genes. Each CT value used for these calculations was the mean of triplicates of the same reaction. Relative RNA levels were expressed as n-fold variations compared with water controls (or as percentages of the average control groups.) All primers used were designed by Eurofins genomics. Sequences of all used primers are listed in **Table 1**.

6.14. Protein extracts and immunoblotting

The protein extract was prepared from the first distal cm of colon by fast homogenization in Tissue Lyser (Quiagen) in an appropriate amount of T-PER Tissue Protein Extraction Reagent solution (Thermo Scientific) containing 0.5 M EDTA, protease and phosphatase inhibitor cocktail (Halt^{TM}, Thermo scientific). The protein extract was subsequently separated by centrifugation at 13 000 RPM at 4°C for 10 minutes. Total protein concentration was measured using Bradford protein assay (Biorad). Protein samples (25 μg) were separated by SDS-PAGE using 12% Bis-Tris Gels (Novex, Life technologies) and blotted onto PVDF membrane (Biorad). The membrane was blocked and incubated overnight (ON) with rabbit monoclonal antibody against PER3 (Abcam, ab177482, 1:1000) at 4°C. Following ON incubation, the membrane was washed and incubated with secondary horseradish peroxidase (HRP)-conjugated antibody (goat anti rabbit, Abcam, ab97051, 1:5000). The signal was visualized using chemiluminescence reaction (Lumi-Light Western Blotting substrate, Roche) and imaged with Vilber Fusion FX. The membrane was thereafter re-stained with primary antibody against HSP 60 (SantaCruz, sc-1052, 1:1000) and visualized as

described above. Digitalized images were quantified using ImageJ software (NIH). The integrated optical density was normalized to the loading control (HSP 60). The data were statistically evaluated using One-way ANOVA followed by Bonferroni's multiple comparison test.

6.15. Cytokine Quantitation in serum (Luminex technology)

Blood samples were collected in EDTA tubes and centrifuged at 1000g (4°C) for 10 min. Serum fraction was collected and frozen at -80°C . Before running the assay, samples were spin down at 1000g 10 min (20-25°C). Cytokine levels were measured using a magnetic bead-based ProcartaPlex Immunoassay kit (Affymetrix, ebioscience) on a Luminex 200 analyzer (Millipore, USA). Samples and antigen standards were prepared in accordance to manufacturer instructions. Briefly, 10 µl of serum were added and incubated with specific antibody-immobilized beads with agitation on a plate shaker overnight at 4°C. The plate was then washed with 200 µl of wash buffer/well three times, and 50 µl of detection antibodies were added to each well and incubated for 30 min with agitation. A further 50 µl of streptavidin-phycoerythrin were added to the well and incubated for another 30 min. The plate was washed, and 100 µl of sheath fluid was added. The median fluorescent intensity was measured on a Luminex Analyzer Bio-Plex 200 system (Milipore, USA), and data were acquired with Bio-Plex Manager 5.0 software (Bio-Rad Laboratories, Hercules, CA).

6.16. Statistical analysis

The arithmetic means +/- SEM values were calculated for all parameters obtained from at least 3 independent experiments and analysed using GraphPad Prism 5.0 and 7.0 (GraphPad Software). To assess the statistical significance for the selected parameters of interest between experimental groups, a one-way ANOVA was used for one variable, followed by a Bonferroni post-hoc test to decrease the likelihood of a rare event in multiple comparisons. In the case of two variables, a two-way ANOVA test was performed, followed as well by a Bonferroni post-hoc for multiple comparisons. *P* values less than 0.05 were considered significant. Correlation between blood glucose and body weight change was analysed by linear regression analysis. Results were considered significant when $p<0.05$.

6.17. Other

Table 1. List of SYBR green murine primer sequences

Gene	Forward primer 5'→ 3'	Reverse primer 5'→3'
Hprt1	TCCTCCTCAGACCGCTTTT	AGGTATACAAAACAAATCTAGGTCAT
Eef1a1	AAGCCCATGTGTGTTGAGAG	CTCCAGCAGCCTTCTTGTC
Clock	GAAGTTAGGGCTGAAAGAC	GATCAAACCTTTCCAATGC
Bmal1	GACAAAGATGACCCTCATG	CATGTTGGTACCAAAGAAG
Npas2	CGCAGATGTTCGAGTGGAAAG	GTGCATTAAAGGGCTGTGGAG
Per3	CCTATTGATTTCCCACCTTCC	CAACAGCACTGGGTAAGAAGCTC
Dbp	TAGAAGGAGCGCCTTGAGTC	GCAACCCTCCAGTATCCAGA
Hlf	GCGTGCTCAGGTCCCTGCTG	AGGCCCCAGGAATGCCGACT
Tef	GCTCTCCAGTGTGCCAGGCG	CGGGCATCCCGTGACCGTTT
Rorc	CTCATGACTGAGAACTTGGCTC	ACCTCCACTGCCAGCTGTGTGCTGTC
Nampt	GACCTGAGACATCTGATAGTATCG	GGAAACTTCTTGCCTAAAATATCTAAG
Irakm	GTTCTACTCCTGTTCCGTCACC	GTCCCGTTGCTCATATAGGGATA
Myd88	TCATGTTCTCCATACCCTTGGT	AAACTGCGAGTGGGGTCAG
G-csf	GGAGCTCTAAGCTTCTAGATC	TAGGGACTTCGTTCCTGTGAG
Gm-csf	AGATATTCGAGCAGGGTCTAC	GGGATATCAGTCAGAAAGGTT
M-csf	CCCATATTGCGACACCGA	AAGCAGTAACTGAGCAACGGG
C-ebp-β	GGTTTCGGGACTTGATGCA	CAACAACCCCGCAGGAAC
Irf8	GAGCGAAGTTCCTGAGATGG	TGGGCTCCTCTTGGTCAT
Bcl3	CCTTTGATGCCCATTTACTCTA	AGCGGCTATGTTATTCTGGAC
Socs3	GGGTGGCAAAGAAAAGGAG	GTTGAGCGTCAAGACCCAGT
Pu.1	CCTCAGTCACCAGGTTTCCTACA	CTCTCACCCTCCTCCTCATCTG
Tnfr1	GGCAGTGCATACCTGTTTTTG	AACCGCAACTGGACGATGAG
Cxcl12	TGCATCAGTGACGGTAAACCA	CACAGTTTGGAGTGTTGAGGAT
TNF	GAAGATGTGCCTGTCCTGTGT	CGCTCAGGTCAGTGATGTTAA
IL-1-β	AAACAGATGAAGTGTAGG	TGGAGAACACCACTTGTT
IL-6	TGGCAAAGGCAGTGTAACTC	CCGGAGAGGAGACTTCACAG
IL-17A	GCTCCAGAAGGCCCTCA	CTTTCCCTCGCATTGACA
IL-10	TGAGGCGCTGTCGTCATCGATTTCTCCC	ACCTGCTCCACTGCCTTGCT

Table 2. List of monoclonal antibodies anti-mouse

Surface anti mouse m-Ab (1:400)		
Antigen	**Conjugation**	**Company**
CD45	APC-Cy7	BD bioscience
CD11b	Percp-Cy5.5	ebioscience
CD11c	PE-Cy7	BioLegend
I-A/I-E	Alexa fluor 700	BioLegend
CCR7	APC	ebioscience
CD86	efluor 450	ebioscience
Ly6C	FITC	BD bioscience
Ly6G	V450	BD bioscience
F480	BV605	BioLegend
CD115	efluorPE-610	BD bioscience
CD64-PE	PE	Biolegend
CD8-a	APC	Biolegend
CX3CR1	PE	RDsystems
Mac3(CD107b/LAMP2)	PE	ebioscience
Linage postitive	PE	ebioscience
Sca-1	PE-Cy7	ebioscience
C-kit	efluor 450	ebioscience
CD127	Alexa fluor 700	ebioscience
CD34	FITC	ebioscience
CD16/32	Percp-Cy5.5	ebioscience
Live/dead far red	APC-Cy-7	Invitrogen
Intracellular anti-mouse (1:400)		
Antigen	**Conjugation**	**Company**
Rorc	APC	ebioscience
IRF8	PE	ebioscience
IL-10	APC	ebioscience
TNF-a	efluor450	ebioscience
IL-17A	PE	ebioscience
Foxp3	FITC	ebioscience
Il17A	PE	ebioscience
IIFN-g	PE-Cy5.5	ebioscience

Table 3. Buffers and medias

Red blood lysis buffer (RBC)
4.15g NH4Cl
0.55g KHCO3
0.185 g EDTA
+Add 500 mL H2O bidest, sterile filtration
MACS buffer
PBS (phosphate saline buffer) pH 7.4 (Gibco)
5g of bovine serum albumin (BSA, Sigma)
4 mL of 0.5 EDTA
FACS buffer
PBS pH 7.4 (Gibco)
2% Fetal Calb Serum (FCS) (Chemie Brunschwig)
0.005% sodium azide
Complete medium
RPMI 1640 (Pan, Biotech) or DMEM (Gibco) or IMDM (Gibco)
10% FBS (Chemie Brunschwig)
Penicillin/Streptomycin, 1% (1000 U/mL and 1000 ug/ mL, respectively)
L-Glutamine (final.conc. 2mM)
B-Mercaptoethanol (final.conc. 0.5 mM)
Percoll gradient for mononuclear cell isolation.
90% Percoll solution (isotonic): 9/10 100% Percoll Stock + 1/10 10x PBS (-/-)
40% Percoll solution: 1/3 90% Percoll + 2/3 DMEM FBS 10% (or PBS 1x -/-).
80% Percoll solution: 3/4 90% Percoll + 1/4 mL DMEM FBS 10% (or PBS 1x -/-).

Table 4. Solutions for LP cell isolation.

Collagenase Digestion solution				
pH 7.2	**Stock**	**In reaction**	**1x, µl**	**3x µl**
Collagenase I*(Worthington Biochem.)	20 U/µl	0.909 mg/ml 300U/ml	45 mg	135
Hyaluronidase (Sigma)	100 mg/ml H2O	2 mg/ml	60	180
Dnase I (Roche)	10 mg/ml H2O	0.3 mg/ml	90	270
CaCl2.2 H2O (In DMEM NO FBS)	stock 70 mM	5 mM	250	750
DMEM (NO FBS)	1x	1x	2600	7800
Total			3000	9000

EDTA solution			
pH 7.2	Stock	In reaction	1x, µl
HEPES	1M	20mM	2000
NaPyruvate	100mM	1.5 mM	300
EDTA, pH 7.2	0.1 M	15mM	3000
HBSS isotonic	10x	1x	2000
FBS			2000
PBS (-/-)	1x	1x	10700
Total			20000

Chapter 7.
References

Chapter 7. REFERENCES

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Chapter 8.

ANNEX

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8.1 Abbreviations

A.

AD Advance-stage disease

AP Active phase

Arg1 Arginase 1

AutoMACS Magnetic Activated Cell Sorting

B.

BCL-3 B cell lymphoma 3 protein

BM Bone marrow

BMAL1 (Arntl) Aryl hydrocarbon receptor nuclear translocator-like protein 1

BMI Body mass index

BW Body weight

C.

CAC Cancer-associated cachexia

CAR Constitutive androstane receptor

CCGs clock-controlled genes

CD Chron's disease

CDK2 or 4 Cyclin-dependent kinase 2 or 4

C/EBP CCAAT-enhancer-binding protein

CFU Colony-forming unit

Ci-sCG Circadian clock gene expression

CLOCK Circadian locomotor output cycles kaput

CLPs Common lymphoid progenitors

CMPs Common myeloerythroid progenitors

CNS Central nervous system

COX2 Cyclooxygenase 2

CP Choroid plexus

CRC Colorectal cancer

CRH Corticotropin releasing hormone

CRY Cryptochrome 1-2

CVOs Circumventricular organs

D.

DAMPs Danger-associated molecular patterns

DBP *D-site albumin promoter-binding protein*

DCs Dendritic cells

DNA Deoxyribonucleic acid

DRRs Danger recognition receptors

DSS Dextran sodium sulphate

E.

ED Early-stage disease

EZH2 Enhancer of zeste homolog 2

F.

FAs Fatty acids

G.

G6PC Glucose-6-phospahte

GC Glucocorticoids

G-CSF Granulocyte colony stimulating factor

G-CSFR G-CSF–G-CSF receptor

GI Gastrointestinal

GIT Gastrointestinal tract

GMPs Granulocyte-monocyte progenitors

GM-CSF Granulocytemacrophage colony stimulating factor

GP2 Pancreatic glycoprotein 2

H.

H3K4 Histone H3 at lysine 4

HAT Histone acetyltransferase

HDAC3 Histone deacetylase 3

HIF-1α Hypoxia inducible factor-1α

HLF Hepatic leukemia factor

HP Hypothalamus

HPA Hypothalamic- pituitary-adrenal

HPCs Highly proliferative lineage-committed progenitor cells

HSCs Haematopoietic stem cells

HSP Heat shock protein intro

HSPCs Hematopoietic stem and progenitor cells

I.

IBD Inflammatory bowel disease

IFNs Interferons

IL Interleukin

IL-10R IL-10 receptor

iMNP Intestinal mononuclear phagocytes

IRAK1 interleukin-1 receptor (IL1R)-associated kinase 1

IRAK-M Interleukin-1 receptor associated kinase-M

IRF Interferon regulatory factor

IRF8 Interferon regulatory factor 8

IκB Inhibitor of nuclear factor

J.

JAK Janus kinase

K.

KO Knock out

L.

LBD Ligand-binding-domain

L-cys L-cysteine

LD Light:dark

L/D Live /dead staining

LP Lamina propria

LPS Lipopolysaccharide

LTi Lymphoid tissue inducer

M.

mABS Monoclonal antibodies

M-CSF Macrophage colony-stimulating factor.

MDPs Macrophage and DC precursors

MDSCs Myeloid-derived suppressor cells

MEPs Megakaryocyte-erythroid progenitors

MHC class II Major histocompatibility complex class II molecule

MLL1 Mixed lineage leukemia 1

MP Myeloid progenitor

MPs Macrophages

MPPs Multipotent myeloid progenitor

mTOR1 Mammalian Target of rapamycine

MyD88 Molecule myeloid differentiation marker

N.

NAD^+ Nicotinamide adenine dinucleotide

NADH Nicotinamide adenine dinucleotide (reduced form)

NAMPT Nicotinamide phosphoribosyl transferase

NETs Neutrophil extracellular traps

Nfil3 Nuclear factor interleukin 3 regulated

NF-k B Nuclear factor kappa light chain enhancer of activated B cells

NO Nitric oxide

NR Nuclear receptor

O.

ONOO− Peroxynitrite

P.

PAMP Pathogen-associated molecular patterns

PAR bZip Proline and acidic amino acid-rich basic leucine zipper

PCG1α Peroxisome-proliferator-activated receptor-ϒ-co-activator 1α

PCK1 Phosphoenolpyruvate Carboxykinase 1

PER Period 1 -3 families

PGE2 Prostaglandin of the E2 series

PMN Polymorphonuclear cells

PPARα Peroxisome proliferator-activated receptor α

PPARγ Peroxisome proliferator-activated receptor-γ

PRR Pattern recognition receptors

PRX Peroxiredoxins

Q.

R.

R. Receptor

RER Respiratory exchanged ration

REV-ERB Nuclear Receptor Subfamily 1 Group D Member

ROR Retinoic acid-related orphan receptor

RORC Receptor nuclear receptor gamma

RORC2 Receptor nuclear receptor gamma isoform 2

RORE ROR response elements

ROS Reactive oxygen species

RP Resting phase

S.

SBS Sickness behavior syndrome

SCN Suprachiasmatic nucleus

SIRT1 Sirtuin1

SOCS3 Suppressor of cytokine signaling 3

STAT Signal transducer and activator of transcription

T.

TAG triacylglyceride

TAMs Tumor associated macrophages

TCA tricarboxylic acid cycle

TEF Tyrotroph embryonic factor

TF Transcription factor

TGF-β Transforming growth factor beta

Th CD4+ T-helper

TIM Timeless

TLRs Toll-like receptor

TNF-α Tumor necrosis factor-α

TRIF TIR-domain-containing adapter-inducing interferon beta

U.

UC Ulcerative colitis

UCPs Uncoupling proteins

V.

W.

WAT white adipose tissue

WT wild type

X.

XPA Xeroderma pigmentosum A

Y.

Z.

ZT Zeitgeber

8. 2. Acknowledgments

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In the first place I would like to acknowledge the funding sources that made possible my PhD work. We would like to thank the Swiss National Science Foundation (grant number 141055), the Lotex Foundation and the Hertie Foundation (both given to my Professor Adriano Fontana). In addition, we would like to thank Dr. Peter Fuchs for his helpful discussions.

I would like to thank Adriano Fontana for giving me the opportunity to be a member of his lab. Throughout my PhD he provided me the chance to work together with a postdoc, Laura Strauss, who guided me through my thesis. You gave us our independence but always tried to keep track of our progress. You showed us to answer one question after the other, to keep the red line and don't get lost trying to assess several subjects at the same time. I will fondly remember our group conversations during the lunchtime and your anecdotes. I also would like to thank you for your support during my health issues during this period.

I would like to thank Laura Strauss for not being merely a 'supervisor', but a colleague with whom I worked 'elbow to elbow' as a team and with whom I generated together most of the PhD data. Without her this work could have not been possible. Although it was not always easy to share a project and often we ended up with big discrepancies, I have to thank her for showing me to be determined and never give up even when your battery is off and you don't have even time to supply it. Thank you for all these time together, for our interesting discussions about science and everything else.

I also want to thank my PhD committee members, Burkhard Becher and Gerhard Rogler, who gave me good advices and ideas for my project during our meetings. They always were willing to help to resolve several problems which I encountered while working on my project. Even if I did not really used that 'card', I really appreciate it. Thank you.

I would like to thank Charlotte Ruhnau, my long-time colleague, who introduced me to the Fontana lab and to the institute and who has become one of my best friends here. Thank you very much not only for our scientific discussions but for your help in resolving problems which I encountered while working on my project and for giving me a hand with the German.

Thank you Lukas for the great time in the lab and for all your experimental help and discussions. Although you arrived quite late to the lab, I really appreciated your presence from the beginning. Thank you for being always direct and honest, and for all our conversations in the balcony.

I also would like to thank the former Fontana members to whom I had the chance to work with, especially Martin. Thank you for being always helpful and willing to solve any problem in the lab. I will never forget your often 'eccentric' behavior and your black humor. Thank you for all our discussions and for sharing with me the nice pictures of the Swiss mountains.

I would like to thank Petra Paul, also a very good friend and talented scientist, who gave me good feedback and tips that helped me through several difficulties during my PhD.

In the same way I would like to thank Sonia Tugues, a very good Catalan friend as well, who was always willing to help inside or outside the institute, even with her very tight experimental schedule. Thank you for our corridor conversations, for all your help and support, and of course, for babysitting my cats ☺.

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Of course, I would also like to thank Becher, Münz, Lünemann and the Joller lab members. Big thank you for all the lab help, advices, discussions and for the fun we had outside of the lab.

Furthermore, I would like to thank our secretary Ines Scholz for her support and willingness of help in bureaucratic matters. Thank you for your positive mood and our lunch conversations.

The biggest appreciation goes to all the friends that I have made during my PhD life, including people from and outside of our institute (you know who you are), who were the greatest help in counterbalancing every single problem arising in my work life.

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Thank you to my 'Rajasthan family' in Zürich ☺. I have no words to explain my gratitude for having you in my life.

Thank you, agent David, for being one of the closets friends in Switzerland. For taking care of me whenever I needed you and for introducing me to the lindy world. Thank you for our paragliding adventures, for our special missions and for all our fun moments.

Thank you Lluis for being the best flat mate ever. For your great humor and all your help when I moved here and I had no one else to hold on. Thank you for taking out my crazy laugh whenever we meet.

Thank you to my very good friends that I left back in Barcelona, who have still been there for me despite the distance. Thank you Eva, Natalia, Sandra and Iris for being YOU and for being part of my life.

Thank you Marc for having been always by my side in the difficult situations and for all your support and friendship. I have to thank you for giving me the courage to move to Switzerland, and for the impact and opportunities that this step will give me in life. Although we did not have it easy you never gave up with me. You are a fighter by nature and I'm sure that life will bring you something good, because you deserve it. Just try to avoid getting upset with board games, 'picón';-P.

I would like to thank my family, for their unconditional support. I would have not made it here without you. Thank you for being always there for me and for giving me everything even when you had not much to give. Thank you for your tremendous effort, for the education and the values you gave me, that helped me to be independent and that opened me doors. Thank you for lighting me when I was very lost and for having always faith in me. Thank you for your love and generosity. I am very glad and very proud of you, and I hope at some point I can be able to give you back a part of what you have given me.

Many thanks to the Nescher family for treating me since the first day as part of them, for all their understanding and help during the last steps of the PhD. Thank you very much for your kind treatment and for 'adopting ' my Monsters when I needed.

Finally would like to Thank Manuel for being the best partner one could ever imagine. Thank you for your patience, understanding and your love. Thank you as well for all your support during the writing process, for making me laugh and for helping me counteract the entropy in my life ;-P. Te quiero.

8.3 Curriculum Vitae

Curriculum Vitae

PERSONAL INFORMATION

Name	Carla Sellés Moreno
Contact	Rotachstrasse, 16 8003 Zürich +41 789276381 carla.sm.87@gmail.com Carla.sellesmoreno@immunology.uzh.ch
Date and Place of Birth	February 3rd, 1987, La Seu d'Urgell, Catalonia, Spain
Nationality	Spanish

EDUCATION

02.2013 – present	**University of Zurich, Institute of Experimental Immunology** Switzerland, Supervisor: Prof. Dr. Adriano Fontana PhD Studies in Immunology, Immunology and Microbiology PhD program "Clock gene dysregulation and nuclear receptor expression in DSS-induced-colitis" **Competences:** Animal models of intestinal inflammation; Telemetry; Cellular culture; Multicolor Flow cytometry (FACSdiva and FlowJo); cell sorting; Automacs; RT-qPCR; ELISA; Luminex.
2010 – 2011	**University of Barcelona & Autonomous University of Barcelona,** Barcelona, Spain Master in Immunology "Tolerogenic dendritic cells as a potential therapy for Experimental autoimmune encephalomyelitis and Multiple sclerosis" **Competences:** Functional cell culture assays; Ex vivo cell differentiation; Multicolor Flow cytometry (FACSdiva and FlowJo); Animal models of multiple sclerosis; Inmunofluorescence; Proliferation assays; Histology; Imaging (IVIS); Rotarod performance test. Cellular culture;
2005 – 2010	**Autonomous University of Barcelona**, Barcelona, Spain Bachelor in Biology, speciality in Health

2003 – 2005	**Joan Brudieu High School Institute,** La Seu d'Urgell, Spain High School education in Science and technology Research project: "Genetic Modified Organisms"
1999 – 2003	**La Salle**, La Seu d'Urgell, Spain Secondary school education

RESEARCH RELATED PROFESSIONAL EXPERIENCE

2013 - Current

Tutor for practical training LTK Module 1 (FELASA), UZH

Competences: rodent (mouse and rat) handling, according to the Swiss legislation; restraining methods; Anesthesia (Inhalation / Injection); Blood collection methods; Injections; surgery; Euthanasia.

Module title: LTK-module 1E. Introduction to the Laboratory Animal Science / Introductory Course in Laboratory Animal Science. Accredited by Federation of Swiss Cantonal Veterinary Officers (VSKT) and recognised by Federation of European Laboratory Animal Science Associations (FELASA)

Supervisor: Dr. Philippe Bugnon

Zürich, Swizerland

10/2010 – 12/2013

Research Associate at Immunology Laboratory for Molecular Diagnosis (LIRAD), Multiple Sclerosis group

Keywords: Autoimmunity; Antigen-specific celular therapy; Immune tolerance; Innate and Adaptive immunity; Multiple sclerosis; Experimental Autoimmune Encephalomyelitis.

Competences: Functional cell culture assays; Ex vivo cell differentiation; Multicolor Flow cytometry (FACSdiva and FlowJo); Animal models of multiple sclerosis; Inmunofluorescence; Proliferation assays; Histology; Imaging (IVIS); Rotarod performance test.

Project Title: Cell therapy in Multiple Sclerosis, Defining guidelines, dose response and response phenotype.

Supervisor: Dr Eva Martínez-Cáceres
Badalona, Barcelona, Spain

07/2009 – 09/2009

Internship at Immunology Laboratory for Molecular Diagnosis (LIRAD)

Keywords: Diagnostic immunology, Experimental and clinical immune tolerance, Clinical epidemiology

Competences: mononuclear cell isolation; immunofluorescence; multicolor flow cytometry; introduction to clinical-welfare diagnostic techniques (Autoimmunity; immunochemistry; cellular immunology; allergology; immunogenetics/ histocompatibility)

Supervisors: Dr. Raúl Castaño, Dr Med. Eva Mª Martínez Cáceres, Dr. Med. Mª Jesús Martínez Arconada

Badalona, Barcelona, Spain

PRESENTATIONS AND POSTERS

2015	8th MIM retreat , Fiesch, Switzerland (Poster Presentation)
2014	Rodent Telemetry User Meeting . Zurich Integrative Rodent Physiology (ZIRP), UZH, Switzerland (Oral Presentation)
2014	9th ENII/EJI Summer School, Porto Conte (Alghero), Sardinia
2014	Introductory Course in Microbiology and Immunology 2014 Institute of Plant Biology, UZH, Switzerland (Oral presentation)
2012	VI th CONGRESS of Immunology Catalan Society (SCI) and III Joint symposium of Immunology Spanish Society (SEI), Barcelona, Spain (Poster presentation)
2012	School of translational Immunology. COST Action ENTIRE and School of Medicine, University of Belgrade, Serbia (Poster presentation)
2012	8th FENS Forum of Neuroscience 2012, Barcelona, Spain (Poster presentation)
2011	Vth SCI CONGRESS and II SEI joint symposium, Barcelona, Spain (Poster presentation)

HOBBIES

Paragliding, Piano, Taekwondo, Lindy hop, Collegiate shag, Trekking, Snowboarding, Skiing.

OTHER SKILLS

Languages: English (Professional competence); Spanish (mother tongue); Catalan (mother tongue), German (basic knowledge A1.2)

Ofimatics (Windows Office package, OpenSource Package); IOS and Windows operative system

Biostatistics software (Graphpad prism; SPSS)

R programming (basic competence)

Other: Driving License (B); SHV/FSVL Solo pilot Paragliding license; Open Water Diving License

OTHER EDUCATION

2008	Grant from the Education and Science Ministry (MEC) Intensive language immersion course in spoken English Universidad Internacional Menendez Pelayo (UIMP), Madrid, Spain
2007	Grant from the Education and Science Ministry (MEC) English course with a host family, Leicester, UK
2005	Grant from the Education and Science Ministry (MEC) English course with a host family, Norwich, UK
2004	Grant from the Education and Science Ministry (MEC) English course with a host family, Abingdon, Oxford, UK

PUBLICATIONS

1. Mansilla MJ, **Sellès-Moreno C**, Fàbregas-Puig S, Amoedo J, Navarro-Barriuso J, Teniente-Serra A, Ramo-Tello C, Martínez-Cáceres EM. Beneficial Effect of Tolerogenic Dendritic Cells Pulsed with MOG Autoantigen in Experimental Autoimmune Encephalomyelitis. CNS Neuroscience and Therapeutics, Nov 18, 2014.

2. Strauss L*, **Sellés-Moreno C***, Rambousek L, Wong WWL, Isabelle Frey I, Fontana A. Clock gene disruption in inflammatory monocytes from mice with colitis is an adaptive response associated with disease remission. Journal of Chron's and Colitis *Shared first authorship (Manuscript in preparation)

3. **Sellés-Moreno C**, Fontana A, Strauss L. Myeloid clock and inflammatory bowel disease: a regulator for disease flare. (Manuscript in preparation)

REFEREES

Prof. Dr. Med. Adriano Fontana. Institute of Experimental Immunology – Inflammation and Sickness Behaviour responsible (UZH)

Dr. Eva María Martínez-Cáceres. Immunopathology Laboratory (LIRAD, IGTP) responsible and associated teacher of Immunology Department (UAB)

Dr. Marta Vives-Pi. Dr. Marta Vives-Pi. Researcher (IGTP) / Lecturer in Immunology (UAB)

Dr. Josep Manyé Almero. Gastroenterology & Nutrition Research. Leader Researcher at Food Functional Research & Innovation Unit (IGTP).
Professor emeritus Joan Barceló Coll. Vegetable Physiology Department (UAB)